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2014

ISBN: 978-90-367-7442-0
ISBN: 978-90-367-7441-3 (e–book)

Financial support for the publication of this thesis was provided by:
AbbVie BV, AllWeCare medical BV, Almirall BV, Department of Dermatology and Schiting Studiefonds Dermatologie, UMCG, Fagron BV, Galderma, Graduate School of Medicine Groningen (GUIDE), LEO Pharma BV, Medizorg BV, University of Groningen, Urgo medical, Will-Pharma BV

Financial support by Vlinderkind and J.P. Nater Foundation for the research described in this thesis is gratefully acknowledged.

Design & Layout: M.O. Wolf, MOTTOW (mottow.nl), Groningen, The Netherlands

university of groningen

REVERTANT CELL THERAPY FOR EPIDERMOLYSIS BULLOSA

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 17 december 2014 om 9:00 uur

door

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geboren op 5 juni 1984
te Lublin, Polen

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For
my Mom, for teaching me to always ask *why*,
my Wife, for tolerating, supporting and stimulating all my *whys*,
and my Daughter, for becoming my *why*.

LIST OF ABBREVIATIONS

AF	anchoring fibrils
BMZ	basement membrane zone
Col17	type XVII collagen
Col7	type VII collagen
DEB	dystrophic epidermolysis bullosa
DMEM	Dulbecco's modified Eagle's medium
EB	epidermolysis bullosa
EDTA	ethylenediaminetetraacetic acid
GMP	good manufacturing practice
HBEGF	heparin-binding epidermal growth factor
HFSC	hair follicle stem cells
HMGB1	high mobility group box 1 protein
IF	immunofluorescence
iPSC	induced pluripotent stem cell
JEB	junctional epidermolysis bullosa
JEB-gen-intermed	JEB, generalized intermediate (earlier JEB-non Herlitz) type
JEB-gen-sev	JEB generalized severe (earlier JEB-Herlitz) type
JEB-loc	JEB, localized type
lam-332	laminin-332
mAb	monoclonal antibody
MelSC	melanocyte stem cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RDEB	recessive dystrophic epidermolysis bullosa
RDEB-gen-sev	RDEB, generalized severe type
RM	revertant mosaicism
SCC	squamous cell carcinoma
TA	transient amplifying
TALEN	transcription activator like effector nuleases
WAS	Wiskott-Aldrich Syndrom
ZFN	zinc-finger nucleases

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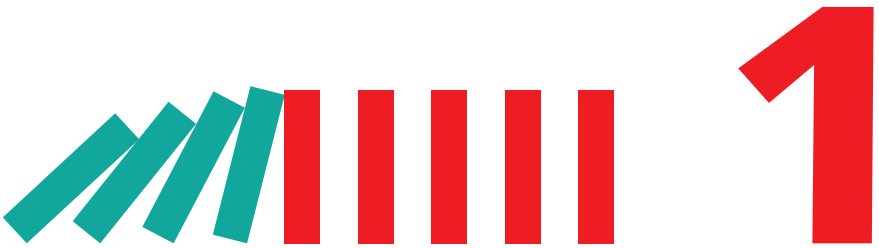

INTRODUCTION

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Fragments of this chapter are a part of the manuscript: Revertant cell therapy for inherited diseases, on the forefront of translational medicine, by Gostynski et al., in preparation.

This thesis describes a quest to understand and use the phenomenon of revertant mosaicism, also known as 'natural gene therapy', to treat the heritable skin disease, epidermolysis bullosa (EB). This introduction chapter can be divided in three parts. We will start with a brief description of the human skin as an organ, followed by details about its two most important cell populations, keratinocytes and fibroblasts. Keratinocyte stem cell biology will be presented in regard to its current clinical applications. Next, a characterization of the dermal-epidermal junction will follow that leads to the description of EB, i.e. the disease caused by mutations affecting proteins involved in epidermal adhesion. Current research on the therapeutic approaches for EB will be briefly presented, which will later allow us to discuss the place for revertant cell therapy. Finally, in the last part the concept of revertant cell therapy will be formulated and at the end of this chapter the aims and an outline of this thesis will be presented.

1

THE HUMAN SKIN

The skin is the largest organ in the human body, weighing approximately 5 kg with an area of 1.5-2 m^2 in adults.[1,2] It forms, together with its appendages, the integumentary system. This system prevents penetration of UV radiation, allergens, toxic substances and other organisms, while also protecting the body from water loss and trauma.[3] The skin also takes part in immune reactions, sensory perception, vitamin synthesis, temperature regulation and secretion of waste. It is also important to mention that the skin plays a role in the social and sexual interaction and communication between human beings.[4]

The skin is composed of two compartments with the epidermis being the outer compartment, and the dermis the underlying connective tissue compartment. These two compartments are connected by the complicated system of adhesive proteins interacting with each other in the basement membrane zone[3] (Figure 1).

Epidermis

The epidermis is a continuously renewing stratified squamous epithelium derived from the ectoderm and averages 50 µm in thickness. Keratinocytes are the major cell population building the epidermis (about 90%) and are organized in four layers representing different stages of differentiation (Figure 1), while on the palms and soles a fifth layer, stratum lucidum, can be found. Aside from keratinocytes, in the epidermis we can find melanocytes, Langerhans cells and Merkel cells.[3] As knowledge about keratinocytes and melanocytes is important for this thesis, those two cell populations will be discussed beneath in more detail.

Keratinocytes

Keratinocytes are named after the keratin intermediate filament protein they contain and are the majority of the cells found in the epidermis. Keratinocytes connect with each other via desmosomes and tight junctions, which provide attachment sites for keratin filaments, present in the cytoplasm and participate in signal transduction and differentiation. The cytoplasm of neighbouring keratinocytes is also channeled by gap junctions, that allow ion and low molecular metabolite exchange.[5] The turnover time of the epidermis, ie. time needed for a keratinocyte to differentiate and migrate from the basal layer to stratum corneum, is about four weeks.[6] The constant renewal, and regeneration following trauma of the epidermis, can be achieved because a population of self renewing epidermal stem cells is present in the basal cell layer.[7]

Epidermal stem cells

Traditionally it has been believed that epidermal stem cells are multipotent, clonogenic and slow cycling. Through asymmetric division they keep their stem cell abilities while giving rise to a

rapidly-cycling transient amplifying cell (TA).[8] In this theory, the stem-cell itself divides rarely, but the TA cell feeds the surrounding differentiated keratinocyte population of new cells ("amplifying"), before final differentiating after a limited number of divisions ("transient").[8,9] There are, however, opposing opinions promoting the theory that a single, constantly cycling progenitor cell gives rise to all the surrounding basal keratinocytes without the TA cells, while maintaining its ability to undergo an unlimited number of divisions.[10] While discussion on the fate of epidermal stem cells is maintained, many groups aim to identify a specific epidermal stem cell marker. At the moment murine epidermal stem cells are believed to express the following surface proteins: Lgr5, Lgr6, CD34, Plet1, Lrig1;[11-15] while also having a high expression of the transcription factor, p63.[16] However, it is still unclear how much of this knowledge can be translated into the understanding of the human epidermis.[8] Expression levels of proteins, such as CD71[17], α6 integrin[18], β4 integrin[19], Lrig1[20], ABCG2[21] or p63[16] can help to identify stem cell rich cell populations. Moreover, high expression of keratins 15 and 19 has also been described in epidermal stem cells.[22] Unfortunately, all these proteins fail to pinpoint a single stem cell and thus cannot be used as a stem cell specific marker.[23]

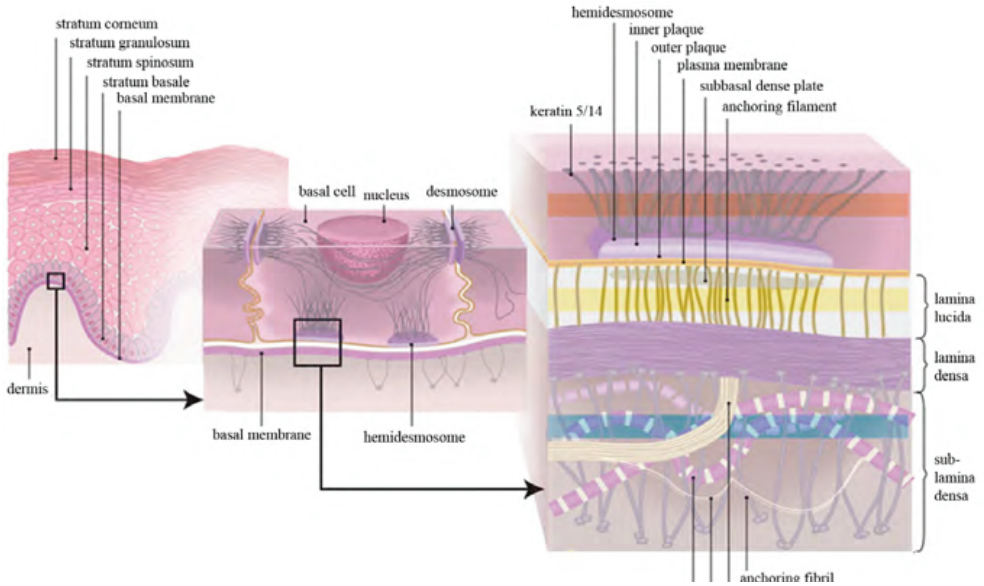


Figure 1 To the left the organisation of epidermis in four layers and basal membrane connecting it to the underlying dermis. In the center we see the magnification of the dermal epidermal junction with the details of the basal membrane zone and hemidesmosome adhesion complex to the right. Courtesy of Prof. dr. M.F. Jonkman[73]

Epidermal cells in regenerative medicine

The history of skin transplantation began in ancient India, where autologous soft tissue flaps were used to cover nasal and ear defects.[24] In 1869 a Swiss surgeon, Jacques-Louis Reverdin, performed the first autologous skin grafting procedure. Since then, autologous grafting of the skin has become widespread and currently the split-skin and full-thickness grafts are a standard procedure.[24] These methods use patients' own skin, which is harvested and then placed on the wound bed, the acceptor site. Autologous skin transplantation can cover areas slightly larger than the donor site, while meshed grafts allow coverage of areas theoretically up to 9 times larger that the donor site but 2-4 fold expansion is more realistic. Unfortunately even such expansion ratios can be insufficient in cases of extensive burn wounds and thus limit donor site areas. The revolutionary work of Rheinwald and Green on culturing human epidermal cells into sheets suitable for grafting resulted in the first transplantation of a cultured keratinocyte graft described in 1981 by O'Connor et al.[25]. Techniques for autologous cultured epithelial autografts were later refined but the technique developed by Rheinwald and Green, involving a feeder layer of murine fibroblasts, still serves as the gold standard.[23] Currently cultured autologous skin grafts are used in treatment of chronic wounds and burns in plastic and reconstructive surgery while also being proposed as the vehicle for genetically modified keratinocytes in gene therapy for genodermatoses.[23,26] Although cultured skin grafts provide the highest expansion ratio, it takes about 3-4 weeks for grafts to be fully grown.[27] This is why other techniques of transplantation of epidermal stem cells, such as enzymatic isolation of epidermal cells and transplantation in the form of cell suspension, are being used for the treatment of chronic wounds, burns and vitiligo.[28-30]

Melanocytes

Melanocytes are derived from the neural crest and can be found in the basal layer of the interfollicular epidermis, in the ratio of 1:10 with keratinocytes, and in the bulb of the hair follicle, where the ratio is lower, 1:5.[31,32] Melanocytes are responsible for production of melanin and transport of melanosomes; organelles containing melanin, to surrounding keratinocytes.[33] Furthermore, the crosstalk between keratinocytes, fibroblasts and melanocytes seem to have an influence on skin homeostasis.[33] Melanocyte stem cells are believed to be in the bulge of the hair follicle, whereas a biologically different melanocyte stem population seems to be present in the dermis.[33] Epidermal stem cells interact with melanocyte stem cells. This will be further discussed in **Chapter 5** and **6** of this thesis. The quest for identification of the melanocyte stem cell is, however, unfinished since a specific human melanocyte stem cell marker has not been discovered yet.

Dermis

The dermis is built from a cellular component, consisting of fibroblasts, macrophages, dendritic cells and mast cells surrounded by the extracellular matrix composed of collagen and elastic fibres, enmeshed by an extrafibrillar ground substance consisting of proteoglycans. Fibroblasts are mesenchymally derived and are the major cell type of the dermis. They are responsible for synthesis of the extracellular matrix. Furthermore, nerves and blood vessels can also be found in the dermis allowing it not only to function as structural support for the epidermis, but also for provision of nutrition.[3]

Basement membrane zone

The basement membrane zone (BMZ) is a sheet-like structure that separates the dermis from the epidermis and is the site of adhesion between these two compartments.[34] The BMZ is a dynamic interface that regulates proliferation, adhesion, differentiation, migration and apoptosis.[35,36] Structures responsible for anchorage of basal keratinocytes to the dermal extracellular matrix are the hemidesmosomes and the focal adhesion complexes[35] (Figure 2). The hemidesmosome complex starts in the basal keratinocytes, where keratin filaments composed of keratin 5 and 14 connect with the inner plaque formed by BP230[37] and plectin.[38,39] It is then connected to the outer plaque, which is built from cytoplasmic domains of type XVII collagen[40] (Col17, also known as BP180) and α6β4 integrin (α6β4).[41] CD151[42] together with the extracellular domains of Col17 and α6β4 forms an extracellular sub-basal plate running parallel to the keratinocyte membrane, while Col17 and α6β4 also connect with laminin-332 (lam-332). Lam-332 together with Col17, laminin-311 and laminin-511 form anchoring filaments, which cross the lamina lucida from the sub-basal dense plate to lamina densa.[38,43] The lamina densa is an electron dense layer that consists mainly of type IV collagen together with nidogen, perlecan and other glycoproteins.[44] The anchoring filaments bind within the lamina densa through the β3 laminin chain of lam-332 to anchoring fibrils (AFs) build from type VII collagen (Col7) that are semicircular structures that extend from the lamina densa to the dermis and then loop back, securing adhesion to the extracellular matrix of the dermis.[45] The focal adhesion complex binds the actin skeleton to the extracellular matrix of the dermis. It starts with the actin microfilament skeleton that binds integrins through kindlin-1. The most important integrin involved in the focal adhesion complex is α3β1 integrin. The extracellular domain of the integrins connect to the laminins, mostly lam-332, which mediates its adhesion to the extracellular matrix of the dermis.[35,46]

Mutations in genes encoding proteins of hemidesmosome and focal adhesion complexes cause epidermolysis bullosa (EB), a group of inherited diseases. In this thesis we will focus on subtypes of EB caused by mutations in genes coding for the Col17, lam-332 and Col7 proteins.

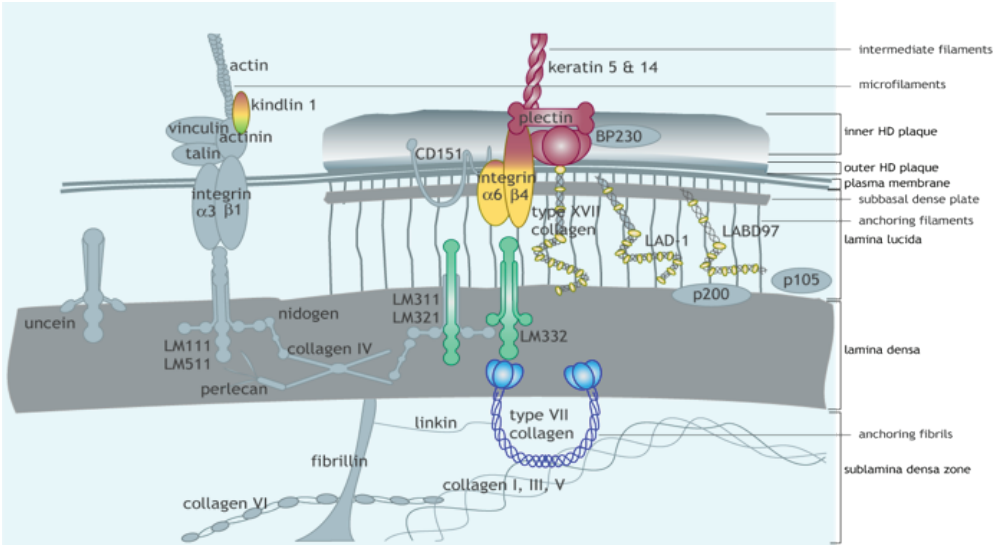


Figure 2 Overview of the hemidesmosome adhesion complex (right) and focal adhesion complex (left) with proteins associated with epidermolysis bullosa in colour. Courtesy of Prof. dr. M.F. Jonkman

Type XVII collagen

Encoded by the gene *COL17A1* that spans 52 kb on chromosome 10q24.3 and consists of 56 exons, the Col17 protein has a molecular mass of 180 kD.[47,48] The *COL17A1* gene is expressed in cornea, teeth, mucous membranes, brain, placenta, umbilical cord and in the skin. It is important to add that in the skin only basal keratinocytes express *COL17A1*.[40] Col17 is a homotrimer built from α1 (XVII) collagen chains of 1,497 residues and has a type II orientation, meaning that the N-terminus (466 residues) starts intracellularly, while the C-terminus (1008 residues) ends extracellularly. The flexible collagen tail domain extends up to the lamina densa.[49] The intracellular domain of Col17 binds to β4 integrin, plectin and BP230, while the extracellular domains binding partner is lam-332.[50,51] Aside from the very important role in dermal-epidermal adhesion, Col17 is important for cell migration and signal transduction.[52-54] Col17 can also be a target for autoantibodies thereby leading to cutaneous pemphigoid, pemphigoid gestationis, lichen planus pemphigoides, linear IgA disease, cicatricial pemphigoid and mucous membrane pemphigoid.[55] Deficiency of Col17 leads to generalized intermediate junctional EB (JEB-gen intermed), which will be discussed later in this chapter.[56]

Laminin-332

All laminins consist of three different laminin polypeptides α, β and γ. Until now, 16 different laminins have been reported, and 5 α, 3 β and 3 γ chains have been identified. Lam-332 is a heterotrimer composed of α3, β3 and γ2 chains encoded by *LAMA3*, *LAMB3* and *LAMC2* respectively.[57] In the epidermis, it is mainly synthesized and secreted by keratinocytes, but actively dividing melanocytes can also secrete lam-332.[58] The assembly of lam-332 starts with the formation of a β3 and γ2 dimer, while the α3 is incorporated later in the endoplasmic reticulum and lam-332 is secreted extracellularly as a heterodimer precursor.[59] Before lam-332 can become a part of the hemidesmosome adhesion complex, it undergoes extracellular proteolytic processing that shortens the 200 kDa α3 chain to 145 kDa and the 155 kDa γ2 chain to 105 kDa, while the 140 kDa β3 chain remains intact.[60] From the epidermal side of the dermal-epidermal junction, lam-332 is connected to α6β4 and Col17, while on the dermal side it connects to Col7 in AFs. Lam-332 is a crucial component of the hemidesmosome adhesion complex.[61,62] Lam-332 is also important for cell migration, in normal epithelial cells, while in invading malignant epithelial cells lam-332 is often overexpressed.[63] The role of lam-332 expression in actively dividing melanocytes has not yet been discovered.[58]

Similar to Col17, autoantibodies can also target lam-332 causing mucous membrane pemphigoid and cicatricial pemphigoid. Lam-332 deficiency causes JEB with the severity depending on the mutation type: nonsense mutations on both alleles encoding one of the chains results in severe generalized junctional EB (JEB-gen sev) that is often lethal,[64,65] whereas presence of at least one missense or splice-site mutation results in non-lethal severe intermediate junctional EB (JEB-gen intermed).[65]

Type VII collagen

The *COL7A1* gene is localized on chromosome 3, p21.31 and consists of 118 exons. Despite the fact that the number of exons is large, the *COL7A1* gene consists of only 31088 bp, and through a cDNA of 8832 bp gives rise to the pro-α1 (VII) peptide, a 2944 amino acid precursor of Col7.[66,67] Pro-α1 (VII) consists of the N-terminal non-collagenous 1 (NC1) domain, triple helix domain in the middle and the C-terminal non-collagenous 2 (NC2) domain. The triple helix domain consists of Gly-X-Y repeats characteristic for collagens, which are interrupted by non-collagenous sequences.[68]

The pro-α1 (VII) peptide undergoes post-translational modifications and three pro-α1 (VII) peptides form a homotrimer, which is secreted to the extracellular matrix. The NC2 domains of the homotrimer are then cleaved by bone morphogenetic protein-1 in exon 115, which allows two homotrimers to dimerize and form AFs with NC1 domains pointing outwards. The NC1 domain contains regions that allow AFs to bind to the lam-332 and integrins, and is thus responsible for

the adhesion of the AFs to the anchoring filaments.[69] Col7 in the skin is synthesized mainly by keratinocytes and, in smaller quantities, by dermal fibroblasts. Col7 is also present in mucosal and bronchial epithelium.[70] Mutations in *COL7A1* lead to different dystrophic subtypes of EB (DEB).[69]

EPIDERMOLYSIS BULLOSA

EB is a group of heterogenic genodermatoses that share blistering of the skin after minor trauma as a symptom. A recent consensus on classification reports that at least 18 genes coding for epi- dermal and dermal proteins (Figure 3) are known to be responsible for causing EB.[65] The level of blister formation in the skin divides EB into four major types but more than 30 subtypes were distinguished based on the affected protein, disease severity, distribution of lesions and presence of extracutaneous manifestations.[65] The annual incidence of EB is about 1 per 17,000 births.[71,72,73]

The severity of clinical manifestations of EB varies from mild to lethal. EB is thus characterized by huge phenotypic variability, depending on the affected gene, type of the mutation, consequence on mRNA, posttranslational modification of expressed protein, and expression level. Apart from cutaneous manifestations, EB can affect gastrointestinal, genitourinary and respiratory tracts; cause growth retardation, anaemia, pseudosyndactyly and increase the risk of developing skin malignancies.[65] Some mutations lead to death within the first few months after birth. According to the newest consensus, the diagnostic process of EB involves identification of the level of cleavage with immunofluorescence (IF) antigen mapping and/or transmission electron microscopy on fresh blisters. Furthermore, immunofluorescence staining for specific antigens is used to identify the affected protein, assess expression level and therefore help to determine the subclassification. When the candidate protein is identified, mutation analysis is performed and the candidate gene is sequenced to allow final subclassification and help with genetic counselling.[65] IF and mutation analysis are also very important in the identification of revertant mosaicism, which will be the subject of the next part of this introduction and will lead directly to the aims of this thesis. However, before we move forward, two major subtypes of EB, junctional and dystrophic EB, will be described in more detail.

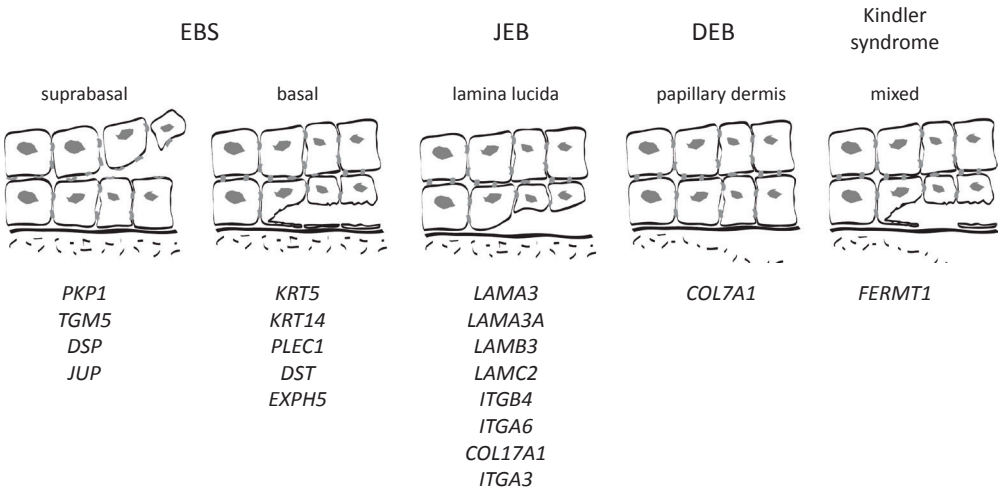


Figure 3 Graphic representation of the level of blister formation in different subtypes of epidermolysis bullosa: EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome. Below the genes involved in the corresponding subtype are listed. Courtesy of Prof. dr. M.F. Jonkman

Junctional epidermolysis bullosa

JEB is caused by mutations in genes encoding the following proteins Col17 (*COL17A1*),[74] lam-332 (*LAMA3*, *LAMB3* or *LAMC2*)[75-77] or integrins, α6β4 (*ITGA6* and *ITGB6*) and α3 subunit (*ITGA3*). Clinical phenotypes are for the large majority inherited autosomal recessively.[65] In JEB, blistering occurs within the lamina lucida of the BMZ. According to the recently published classification, JEB is divided in a generalized type (JEB-gen), with widespread blistering and a localized type (JEB-loc) with milder blistering, often only on hands and feet, and not affecting hair growth. Within the JEB-gen subtype, the form caused by total absence of functional lam-332 earlier named JEB-H is now named JEB-generalized severe (JEB-gen sev).[65] When a child with this form of EB is born, the blistering of the skin and mucous membranes results in complications such as anemia, dyspnoea and failure to thrive, which are so severe that they lead to death with an average life expectancy of 6 months.[64] The other main form of JEB earlier named JEB-nH can be caused by reduced levels of lam-332 or absent levels of Col17 and causes severe blistering and is now named JEB-generalized intermediate (JEB-gen intermed). Patients with JEB-gen-intermed and mutations in lam-332 have an increased risk of developing cutaneous squamous cell carcinoma (SCC).[78]

Dystrophic epidermolysis bullosa

All subtypes of DEB are caused by mutations in the *COL7A1* gene, which leads to defective and/or reduced numbers of AFs.[79] DEB can be inherited both autosomal recessively and dominantly. Blistering in DEB occurs below the BMZ, in the papillary dermis, which in contrast to other forms of EB, can lead to scarring. Severity of recessive DEB (RDEB) depends on the mutations combinations, which can result in different amounts of Col7 expression.[80] Disease subtype correlates with the amount of Col7 present in the dermal epidermal junction when determined by immunofluorescence.[80]

Patients with RDEB experience spontaneous blistering after the slightest mechanical stimuli and heal with extensive scarring resulting in fibrotic scarring, atrophy of the skin and often loss of hair follicles. Of all patients wtih EB, RDEB generalized severe (RDEB-gen-sev) patients experience the poorest quality of life. Characteristics symptoms of RDEB-gen-sev are pseudosyndactyly, a gradual fusion of fingers and toes, leading to deformation of hands and feet, microstomia, dysphagia, anemia and growth retardation. Long-term complications of RDEB-gen-sev are renal failure and development of highly aggressive SCC, which results in death of the majority of patients before 40 years of age.[81]

THERAPEUTIC APPROACHES FOR EB

At the moment there is no cure for EB.[82] There are therapies that have great potential to become one in the future and there are therapies that ameliorate the course of the disease. This thesis focuses on the quest to establish a therapeutic approach using the phenomenon of revertant mosaicism in EB. As revertant mosaicism and the concept of the revertant cell therapy will be discussed in the next part of the introduction, it is of importance to first introduce strategies that are currently under investigation to cure EB.

When discussing therapy the therapy for a genetic disease, three main groups can be identified:[83-85]

- Gene therapy wich aims to introduce a wild-type copy of a gene into the patient's body, which will result in a wild type phenotype. It can be achieved *ex-vivo/in-vitro* or *in-vivo*.
- Protein therapy, which aims in supplementation of the missing or non-functional protein with a protein obtained from healthy donors or a recombinant one.
- Cell therapy, which encompasses therapeutic approaches using allogeneic or autologous cells to directly or indirectly reinstate the missing or non-functional protein.

All three above mentioned strategies are being developed for EB. We will briefly describe the idea behind each strategy, while the advantages and disadvantages of each approach will be described and debated in the discussion chapter **(Chapter 6)** of this thesis.

Gene therapy for EB

Gene therapy for EB aims to correct the affected gene in the epidermal cells or introduce the wild-type copy into the keratinocytes. The best illustration of how this approach works, is the only published successful case of gene therapy in a patient with EB. In 2006 Mavillo et al. reported the successful treatment of a JEB-gen-intermed patient with mutations in *LAMB3*.[26] Using a murine leukemia virus based retroviral vector expressing long terminal repeat driven *LAMB3* cDNA, patient's keratinocytes containing epidermal stem cells, earlier isolated from a skin biopsy, were tranduced *ex-vivo* and transgenic epidermal grafts were cultured *in vitro*. Affected epidermis on the patient's upper legs was removed surgically and cultured grafts were placed on the wounds. In 2014 a follow-up report showing stable expression of lam-332 in the transplanted regions after 6.5 years (80 complete renewing cycles of the transplanted epidermis) was published.[86] Further clinical trials were, however, stopped due to safety concerns by the regulatory committees, as possibilities of random genomic integration of the viral vectors and its consequences deemed to be better understood.[87] Since 2006, many more viral vectors, not only for *LAMB3* but also for other EB genes, for example *COL7A1* were introduced and usage of viral vectors became very efficient to correct the defect in patient cells.[88,89] More data about safety of viral vectors have been acquired and clinical trials will follow shortly.[87] Recently however, techniques allowing in situ correction of the genome using genome-editing strategies, as zinc-finger nucleases (ZFNs) or transcription activator like effector nuleases (TALENs), have been introduced to remove the risk of insertional mutagenesis.[90] TALENs for example have been used successfully to correct *COL7A1* in human fibroblasts *in vitro,*[91] but this approach is still far from clinical trials.

Protein therapy

Substitution of the missing or non-functional protein in genetic disorders of enzyme production has been already used in situations, where the affected cells can take up the enzyme. Recent studies showed that employment of protein therapy in cases of defective structural protein could also lead to promising results.[92,93] Intradermal injections of recombinant Col7 resulted in the incorporation of Col7 in wounded skin, but not in internal organs and unaffected skin.[94,95] As intradermal injections can be painful and, even if Col7 is a very stable protein, would have to be repeated a better systemic approach is needed. Col7 is a soluble protein, thus systemic, intravenous application is feasible. Woodley et al. recently reported restoration of anchoring fibril formation and dermal-epidermal adherence in a murine model of RDEB by intravenous injection of recombinant Col7.[96] Although this approach seems very close to clinical translation, other groups have raised safety concerns regarding the possible immunological response to recombinant Col7.[87,97]

Cell therapy

Autologous skin grafting has been used for many years to close wounds in patients with EB.[98-100] In the last decade dispersions of allogeneic and autologous cells were used *in situ* or systemically. Examples are, intradermal injections with allogeneic fibroblasts or with mesenchymal stromal cells, and systemic hematopoietic stem cell transplantation.[83,85,87] Wong et al. showed in 2008 that intradermal injection of allogeneic fibroblasts in RDEB can upregulate *COL7A1* expression for 3-6 months.[101] A study comparing a single intradermal set of injections with allogeneic fibroblasts in and around a skin erosion showed greater reduction of wound area up to day 28 compared to only vehicle injection, but the difference seemed to be gone at the 6 months timepoint.[102] This study also failed to show statistical significance between the fibroblast and the vehicle group, probably due to small number of treated lesions. The compared group showed no difference between fibroblasts and vehicle injections with both significantly accelerating the wound healing process and similar expression of Col7.[103] These results suggest that fibroblasts themselves may not be crucial for the positive effect on wound healing but the upregulation of *COL7A1* by the heparin-binding epidermal growth factor-like growth factor (HBEGF) could be the mechanism of action.[102,103]

In 2007 a study with bone marrow transplantation for RDEB began in Minnesota, USA and was published in 2010.[85,104] Children with RDEB received myelo-ablative chemotherapy and transplantation of allogenic stem cells from a related HLA matched donor. Results from this trial with seven patients included, and six that completed the treatment, were promising. Patients showed improvement in the phenotype and donor cells could be identified in skin and mucosa. It is, however, important to add that none of the patients was cured completely and while amelioration of the disease was achieved, results varied significantly between patients, with Patient 1 having only modest benefit and Patient 7 having recurrence in blistering after 60 days. Moreover, from seven included patients, one child died during the aggressive conditioning regimen, and one 183 days after the transplantation from subsequent infections showing mortality of this treatment to be above 25%. Mortality rates in bone marrow transplantation vary between indication, conditioning protocol and experience of treating center but for the mixed population with benign disorders and with reduced toxicity conditioning is estimated at around 15-20%.[105] Therefore the usage of systemic stem cell application should be further investigated and modified to achieve lower mortality rates. An approach with reduced toxicity conditioning together with the use of umbilical cord blood and additional infusion of mesenchymal stromal cells instead of bone marrow has been proposed.[87]

A place for revertant cell therapy

Despite the fact that many therapies for EB are in development, none of the proposed approaches has become a cure yet. For example, very promising gene therapy is impeded by safety issues and regulatory bodies while offering only treatment of a limited area of the body, while stem cell therapy in the form of hematopoietic stem cell transplantation using bone marrow does not completely cure the disease and has a high mortality rate. There is, therefore still a place for a safe and successful therapy for EB. In the next part of this chapter we will introduce the phenomenon of revertant mosaicism, a starting point for development of revertant cell therapy.

REVERTANT MOSAICISM – A NATURAL GENE THERAPY

In monogenetic disorders, mosaicism is a co-existence of two different cell populations within one organism, one containing a wild-type gene and one containing a mutated, disease causing, version of the same gene in one individual.[106] Mosaicism is usually an effect of a somatic mutation that occurs post-zygotically in one cell, which later gives rise to a larger cell population that can be identified.[107] Somatic mosaicism can be divided in forward and revertant mosaicism. We speak of forward mosaicism, if in a healthy organism a population of cells becomes affected by a genetic disease as a result of a somatic mutation that occurred post-zygotically. Revertant mosaicism (RM), however, describes the opposite situation, where a somatic mutation restores a wild-type phenotype in a population of cells in a diseased organism affected by a genetic disease. Thus forward somatic mosaicism induces a diseases phenotype in a cell population of a further healthy organism, while revertant mosaicism restores the healthy phenotype in an already affected organism (Figure 4) and therefore can be named natural gene therapy. Forward mosaicism as a cause of genetic disorders has been hypothesized for many years but proof of its existence at the DNA level only came in 1988 in the case of a boy with mild expression of ornithine transcarbamylase deficiency.[108] Many cases have been already described and forward mosaicism has been widely accepted as one of the origins of genetic diseases.[109] Recently, forward mosaicism was demonstrated in the mother of a child with DDEB.[110]

Revertant mosaicism is, regardless of increase in number of described cases, still thought to be a rare phenomenon. It has been observed for ages in plants, not as natural therapy of genetic disorders but as a variation of their color and symmetry. For example, the yellow color of maize is an effect of mutation inbreed, while the natural color is purple. One can quite often observe single purple segments or even spots within one segment, a result of reversion to the wild-type genotype by transposons.[111] The first case of revertant mosaicism as a mechanism of natural gene therapy leading to reversion to a wild-type phenotype in a human affected by genetic diseases was described in 1988, when the reverted HPRT gene was found in a patient with

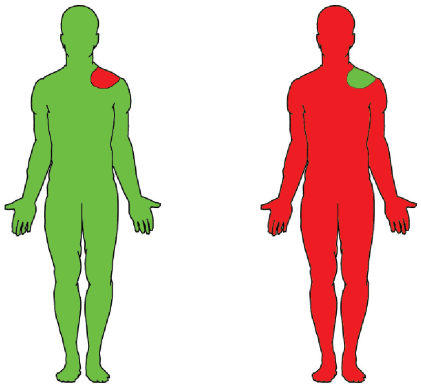
Lesch-Nyhan syndrome.[112] Since then, reversions have been found in other genetic diseases, and are observed in self-regenerating organ systems such as liver, blood and the skin.[113,114]
Reversion is a result of a genetic event and can occur due to different genetic mechanisms as gene conversion, intragenic (single and double) crossing over, back and second-site mutations. Second-site mutations can vary from single base pair substitutions, deletions or insertions to a large deletion of more than 2000 bp (Figure 5). As an effect of the second genetic correcting event, a wild-type protein, a protein with a different amino acid, or an aberrant, but partially functional protein, can be expressed. Surprisingly, more than one reversion mechanism can be present within a single individual. For example in one Wiskott-Aldrich Syndrom (WAS) patient due to a nonsense mutation in the WASp gene, 38 different reversion mechanisms were found[115]. The trigger for mutational DNA events leading to reversion is unknown.
The incidence of revertant mosaicism varies from around 11% in WAS,[116] 18% in Fanconi anemia,[117] 88% in tyrosinemia type I,[118] to presumably 100% in the subtype of JEB-nH caused by mutations in *COL17A1*.[119] Furthermore, additional cases are being constantly described, as recently in dystrophic EB (DEB),[120-122] ichthyosis with confetti[123] or X-linked lymphoproliferative disease.[124] This means that the realm of revertant mosaicism is expanding and its position changes from an exceptionally rare phenomenon towards a common aspect of genetic diseases.[125]

Revertant mosaicism in EB

The first case of a mosaic pattern in the skin was published in 1995 and described a woman, then 27 years old, affected by JEB-gen-intermed due to mutations in *COL17A1*, with patches of healthy looking skin distributed on her arms and hands.[56] The healthy looking skin could also withstand friction as opposed to its surrounding skin, where blisters could be easily provoked (Figure 4B). Immunofluorescence staining showed total absence of Col17 in the biopsy taken from the affected skin, while in the biopsy taken from one of the healthy looking patches, a linear staining for Col17 was seen in about 50 % of the basal cells (Figure 4B). Two years later, in 1997, Jonkman et al. found mitotic gene conversion to be the underlying mechanism of the phenomenon named revertant mosaicism.[126] The patient was heterozygous for a maternal frame shift mutation in exon 18 (c.1601delA) and paternal nonsense mutation in exon 51 (c.3676C>T). Most probably during mitosis of an epidermal stem cell a part of the paternal allele of *COL17A1* containing a healthy copy of exon 18 was moved to the maternal allele and covered the c.1601delA mutation. This resulted in one allele with a paternal mutation and one wild-type allele present in one of the cells present after the mitosis, which later gave rise to the patch of revertant skin.[126]

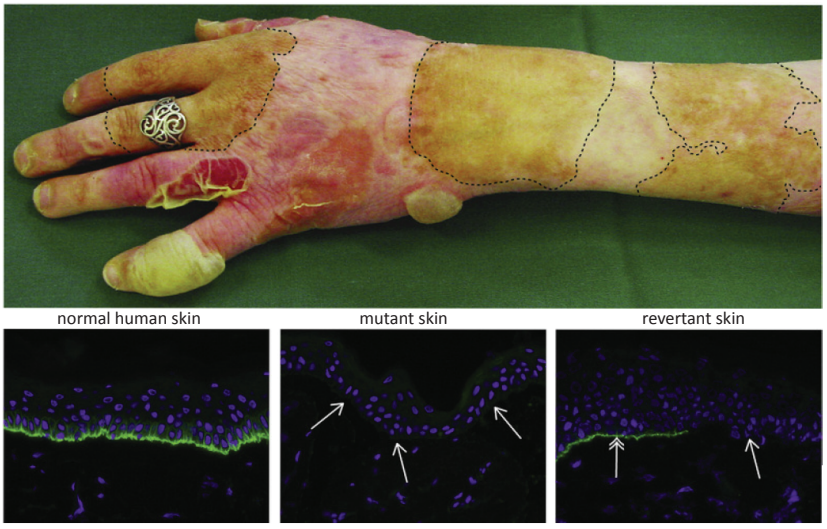
Figure 4 Graphical representation of somatic mosaicism and difference between forward and revertant mosaicism (A). On the left picture a skin patch with mutation (red) is present in an otherwise healthy body with a wild-type genotype (green). On the right picture situation is reversed, as an individual affected by genetic disease (red) has a healthy patch caused by revertant mosaicism (green). (B) shows a clinical picture of the patient with mutation in *COL17A1* presenting healthy looking, hyperpigmented skin patches (outlined) on her left arm surrounded by affected, blistering skin. Below pictures showing immunofluorescence staining for Col17, nuclei staining in blue and Col17 in green on all three pictures. On the left staining of a healthy control human skin showing normal expression of Col17; in the middle staining of the biopsy taken from this pateints affected skin with absence of Col17 (arrows) and to the right staining of the biopsy taken from the healthy looking skin with the partial expression of Col17 (double arrowhead). Part B of this figure is reproduced from Pasmooij et al. 2012 [129]; permission was obtained.

A



forward mosaicism revertant mosaicism

B



normal human skin mutant skin revertant skin

Current knowledge about revertant mosaicism in EB

Almost 20 years later, in 2014, from 18 genes causing EB, five already have been found to be reverted, totalling 34 described and published cases. An overview of the patients with revertant mosaicism per subtype of EB and affected gene is given in Table 1. With number of patients grew the number of different reversion mechanisms identified. Moreover, as in WAS, EB patients were found to have more than one revertant mechanism present in their body and each reversion event lead to a distinct revertant patch on the skin.[127] Furthermore, none of the four major types of revertant mechanisms found in EB seems to be favoured. In the revertant skin patches of EB patients, only keratinocytes were found to be revertant, whereas fibroblasts kept their mutant genotype.[128,129] There is still a lot to be discovered about RM, such as time of onset, possible growth of revertant skin and if some genes have a predisposition to become reverted.

In theory, a reversion in a stem cell during embryotic development would lead to a patchy skin pattern after birth with the same reversion mechanism present in each patch. Such a case, however, has not yet been described. If reversion occurs after diversion of an epidermal lineage, the earlier it happens, the larger the area of revertant skin should be, as it would grow exponentially with the skin. Interestingly, some patients reported that their skin healed later in life, one of them being a patient with *LAMB3* mutation, who indicated that his leg, earlier affected, became healthy during adulthood.[128] Beside the number of divisions of the reverted cell, one has to also consider the possible growth advantage or disadvantage. In the mouse model of EB simplex caused by a mutation in the gene encoding the epidermal intermediate filament, keratin 14 (KRT14) cells with the induced KRT14 mutation were overgrown by healthy keratinocytes suggesting that the healthy cells had an *in vivo* growth advantage.[130,131] It is therefore believed that for a revertant patch to become visible and recognisable, the following conditions have to be met:

- reversion must take place in an epidermal stem cell,
- reversion needs to occur early enough during epidermal development to have a chance to grow to a patch of visible size and/or growth of revertant cells *in vivo* is speeded by its biology.

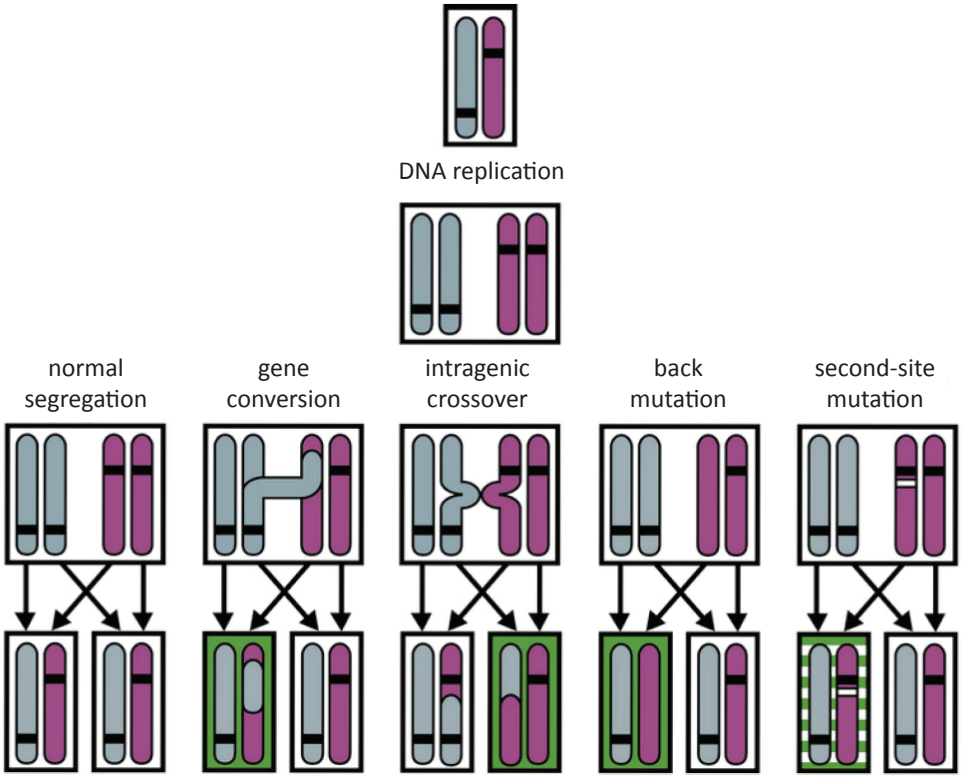


Figure 5 Different reversion mechanisms can correct an inherited mutation. In recessive disease, every cell contains two chromosomes, and the black bars indicate the positions of the mutations. With normal segregation each cell obtains one chromosome from the father and one from the mother. When DNA from one chromosome is non-reciprocally transferred to the other chromosome gene conversion occurs. In case this gene transfer is in the region containing the inherited mutation this mutation is lost in the daughter cells. One of the daughter cells will only carry one recessive mutation, and will therefore produce protein (green). Other reversion mechanisms, seen in hereditary skin diseases, are intragenic crossover, back mutation, and second-site mutation. Intragenic crossover results in one daughter cell with both inherited mutations on one chromosome, and another revertant daughter cell with one of the chromosomes without inherited mutations. In case of a second-site mutation a compensatory mutation is present in the same gene correcting the inherited mutation. The protein that is produced may be similar to the wild-type protein, or slightly aberrant although still functional (green/with stripes). Reused from Pasmooij et al with permission. Courtesy of dr AMG Pasmooij. [129]

It has also been suggested that no extraordinary mutation rate is needed for the reversion to occur and that the standard mutation rate is sufficient.[132] Studying old photographs of patients and comparing the revertant patches to the current situation have proved the stability of revertant patches in EB.[127] In a few cases, however, the growth of revertant patches have been claimed and Choate et al. showed that new revertant spots can arise during life in a patient with ichtyosis with confetti, disease caused by mutation in keratin 10 gene, KRT10.[123]

Clinical presentation of revertant mosaicism in EB

The presentation of revertant skin differs between types of EB, but lack of blistering is the common denominator. In patients with mutations in *COL17A1*, revertant skin contains hair and is darker than surrounding affected skin. This will be further elaborated upon in **Chapter 5** of this thesis. In patients with mutations in *LAMB3* or *COL7A1* lack of atrophy or erythema is an important sign but no difference in pigmentation can be seen. Revertant patches can also vary in size, from 2 to about 10 centimetres in diameter. The number of revertant patches per patient may also differ, from one to multiple covering up to around 10% of the total body surface. As for now, the youngest patient was 10 years old when his revertant patch was identified.

The concept of revertant cell therapy

Revertant cell therapy is a logical step forward. The cultured epidermal grafts composed of revertant keratinocytes might be used to cover wounds. The technique for cultured skin grafting is available and used for treatment of burns and leg ulcers.[8,27] Keratinocytes, and their stem cells, are affected by mutations causing EB and thus can also become naturally corrected in patients with revertant mosaicism. Therefore in this thesis we propose an approach to treat cutaneous manifestations of EB by autologous transplantation of revertant keratinocytes and their stem cells. This approach does not require genetic manipulation, introduction of recombinant proteins or usage of allogeneic material.

AIMS AND OUTLINE OF THIS THESIS

The general aim of this thesis was to translate the phenomenon of RM in to revertant cell therapy, which could benefit the population of EB patients. In **Chapter 2** we describe a human pilot study of transplantation of revertant epidermal grafts in a patient with JEB-gen-intermed due to mutations in *COL17A1* gene. **Chapter 2** shows an elegant technique to prepare a wound bed of the acceptor site by removing the affected mutant skin using the natural pathological mechanism of the disease that causes splitting of the epidermis in the lamina lucida. The transplantation was surgically successful, but skin fragility did not improve since the graft contained insufficient revertant cells. Decrease in the percentage of revertant cells is the main focus of **Chapter 3**, which describes a murine model of revertant cell therapy. We follow the revertant keratinocytes from the moment that the skin biopsy is taken, through the graft production up to 16 weeks after transplantation on mice. **Chapter 3** gives thus more insight in the reason why the procedure described in **Chapter 2** was not successful and suggests the underlying mechanism. Based on experience from chapters 2 and 3 we then took a step back and used grafting of punch biopsies instead of cultured epidermal grafts in a patient with mutations in *LAMB3* gene. This procedure, described in **Chapter 4**, was successful and led to expansion of the revertant area of the patient's body. Although during the experiments described in **Chapters 2-4** we tried to move from the bench to the bedside, the encountered differences in pigmentation of revertant patches between subtypes of EB made us wonder if proteins involved in the dermal epidermal junction also influence pigmentation. Therefore in **Chapter 5** the translation process was reverted and we moved from the bedside to the bench and looked into the pigmentation and melanocyte distribution in the revertant and mutant skin of revertant patients with mutations in *COL17A1*, *LAMB3* and *COL7A1*, which resulted in interesting findings adding to the understanding of melanocyte biology. **Chapter 6** describes three experiments that did not lead to a publication but could be a basis for future experiments and development of in the development of the revertant cell therapy. Briefly, we used of flow cytometry for selection of Col17 revertant keratinocytes, attempted to transplant Col7 revertant human keratinocytes in a murine model, and performed a clinical pilot to transplant revertant keratinocytes in suspension. Finally, in **Chapter 7** our findings are discussed and confronted with the newest developments in the therapy for EB and regenerative medicine. In this chapter we also propose future studies needed to develop revertant cell therapy, not only for EB but also for other inherited genetic diseases.

TABLE 1

Patients with epidermolysis bullosa and revertant mosaicism. Legend: aa, amino acids; Col7, type VII collagen; Col17, type XVII collagen; KRT14, keratin 14; Lam-332, laminin-332; N/D, not determined. Based on table by Pasmooij et al.,[129] modified and updated.

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
Recessive EB Simplex	KRT14	1	c.526-2A>C; c.526-2A>C	N/D	KRT14 with deletion of two aa and one different aa (p.Ile176Met)	133
EB Simplex generalized severe		1	p.Arg-125Cys	c.242insG	Ablation of KRT14 with the c.Arg125Cys mutation	134
JEB-gen-in-termed	COL17A1	12	c.2237delG; c.2237delG	c.2263+2T>C	Col17 with deletion of 12 aa	56,126, 135-137
			c.1601delA; p.Arg1226X	gene conversion c.3677G>C	wt Col17 Col17 with one different aa (p.Arg1226Ser)	
			c.2237delG; p.Arg1226X	c.2228-101_2263+70del ins15 c.2259_2263+9del c.2263+2T>C	Col17 with deletion of 12 aa Col17 with deletion of 12 aa Col17 with deletion of 12 aa	
			c.2237delG; p.Arg1226X	c.2263+2T>C	Col17 with deletion of 12 aa	
			p.Arg1226X; c.4320insC	3676T>C or gene conversion c.4358-1G>A	wt Col17 Col17 of correct size with a stretch of 13 different aa	

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.2237delG; c.2237delG	c.2227+153_2336-318del c.2238C>T	Col17 with deletion of 36 aa Col17 with deletion of 12 aa	
			c.2237delG; c.2237delG	N/D	N/D	
			c.1179delA; c.3327delT	N/D	N/D	
			c.3131delC; c.3131delC	N/D	N/D	
			c.1260delC; c.3495-3496delCT	N/D	N/D	
			c.3898-3899delTC; c.3898-3899delTC	c.3973-3974dupGG	Col17 with insertion of 25 aa	138
			N/D	N/D	N/D	139
	LAMB3	2	c.628G>A (p.Glu210 Lys); p.R635X	c.596G>C (p.Gly199Ala) c.628+42G>A	Lam-332 with two different aa (p.Gly199Ala and p. Glu210Lys) Lam-332 with insertion of 22 aa	128

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.628G>A (p.Glu210 Lys); c.628G>A	c.619A>C (p.Lys207Gln) c.629-1G>A c.565-3T>C	Lam-332 with two different aa (p.Lys207Gln and p.Glu210Lys) Lam-332 with deletion of 22 aa Lam-332 with one different aa (p.Glu210Lys)	
Recessive DEB	COL7A1	9	p.Arg578X; c.7786delG	intragenic crossingover	wt Col7	120
			c.6527dupC; c.6527dupC	single nt deletion c.6528delT	wt Col7	140
			p.Gln2170X; p.Gln2170X	c.6510G>T	Col7 with one different aa (p.Gln2170Tyr)	122
			c.425A>5; c.8206G>A	Mitotic recombination: c.425A>G absent	Wt Col7	141
			c.2142A>G; c.6527dupC	Second-site mutation: c2144A>G	substitution p.Tyr-715Cys	
			c.884delG; c.6527dupC	Back mutation/mitotic recombination: c.884delG absent	Wt Col7	
			c.425A4G; c.425A4G	Second-site mutation: c.426+3G>A	Wt Col7	

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.425A4G; c.1837C>T	Mitotic recombination is suspected with both mutations present in one allele and other being a wild-type (no molecular proof)	Wt Col7	
			c.2894C>T; c.6176A>G	Back mutation/mitotic recombination: c.6176A>G absent	Wt Col7	
Dominant DEB	COL7A1	1	c.6127G>A; N/A	Back mutation/mitotic recombination: c.6127C>A absent	Wt Col7	141
Kindler syndrome	FERMT1	8	N/D	N/D	N/D	142
			c.676dupC; c.676dupC	transcriptional slippage or RNA editing	Wt kindling 1	143
			c.456dupA; c.456dupA	slipped mispairing and mitotic recombination	Wt kindling 1	144
			c.676dupC; c.676dupC	slipped mispairing and mitotic recombination	Wt kindling 1	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; Trp559X	N/D	N/D	

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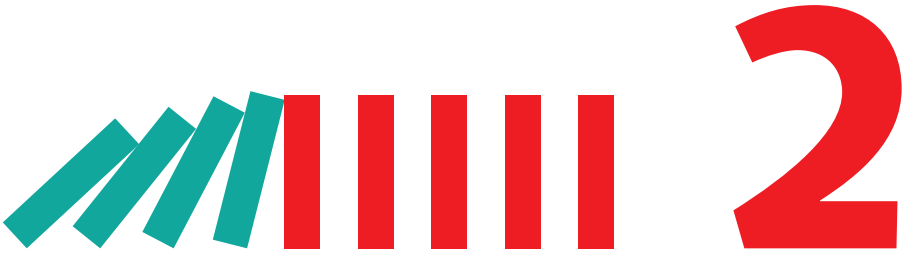

ADHESIVE STRIPPING TO REMOVE EPIDERMIS IN JUNCTIONAL EPIDERMOLYSIS BULLOSA FOR REVERTANT CELL THERAPY

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Published in British Journal of Dermatology 2009 Aug;161(2):444-7

SUMMARY

Background. Replacing mutant skin in epidermolysis bullosa (EB) by epithelial sheets of transduced autologous keratinocytes is the essential surgical step of ex vivo gene therapy. The same applies for *revertant cell therapy* in which epithelial sheets of revertant autologous keratinocytes are used. Revertant cells can be found in patches of normal skin in patients with junctional epidermolysis bullosa (JEB) due to revertant mosaicism caused by *in vivo* reversions.

Objectives. To develop a technique of adhesive tape stripping as a method for epidermis removal to prepare the acceptor site for revertant cell therapy in a patient with revertant mosaic JEB.

Methods. We performed revertant cell therapy on a patient with mosaic type XVII collagen-deficient non-Herlitz JEB. Skin biopsies were taken from revertant skin on the wrist. Graft production took place on a 3T3-J2 feeder layer resulting in two 6x7 cm grafts. An innovative method that uses the pathological plane of least resistance of JEB skin was developed to prepare the acceptor site. A polyacrylate adhesive plaster was placed on the skin and then pulled off with the epidermis.

Results. The epidermis was easily removed with the plaster. The skin separated at the level of the lamina lucida, leaving a bloodless wound bed of naked lamina densa. Transplantation was successful; the acceptor site healed without scarring. However, blistering could be provoked. The functional repair was not achieved due to the low percentage of revertant cells in the graft.

Conclusions. We conclude that adhesive stripping is a simple, effective and almost painless procedure for removing epidermis for ex vivo cell therapy in EB.

Epidermolysis bullosa (EB) is a group of hereditary genetic blistering diseases of skin and mucosae. Junctional EB (JEB) is caused by mutations in the genes for type XVII collagen, laminin-332 or integrin $\alpha 6\beta 4$ which are crucial for keratinocyte adhesion to the basement membrane. Some patients with JEB notice normal, nonfragile skin patches. This phenomenon is called revertant mosaicism.[1] It is caused by naturally occurring somatic *in vivo* reversions that restore protein function and provide restoration of the keratinocyte adhesive function. Skin that contains sufficient revertant keratinocytes displays a wild-type phenotype.[1]

Most studies of cell therapy for EB focus on restoring function by adding correct genes or repairing defective genes of mutant stem cells, and growing these into transplantable epidermal sheets.[2] In revertant cell therapy there is no need for corrective gene manipulation. Epidermal sheets for transplantation are produced from autologous revertant keratinocytes that are present in the healthy skin patches. An effective transplant technique is important for revertant cell therapy to avoid scarring and patients' discomfort.

Here we report on the innovative technique of adhesive tape stripping as a method for epidermis removal to prepare the acceptor site for reverant cell therapy in a patient with revertant mosaic JEB.[1,3]

Patient and methods

The patient had JEB of the generalized non-Herlitz type due to compound heterozygosity of *COL17A1*: c.1706delA and c.3781C>T,p.R1226X mutations in the gene for type XVII collagen. About 4% of her total body surface was revertant due to at least two different *in vivo* reversions: a gene conversion that corrected the 1706delA germline mutation (arm, forearm, hand) and a second-site mutation 3782G>C (ankle) that prevented the premature ending of translation by the R1226X germline mutation.

The left wrist was selected for the donor site, and the upper right leg was chosen for the acceptor site. Keratinocytes were harvested from a 6-mm punch biopsy by enzymatic digestion and cultured on a feeder layer of irradiated mouse fibroblasts (3T3-J2 cells). The cells grew slowly and it took five weeks to grow two epidermal sheets of 6x7 cm each. The sheets were clipped to synthetic gauze to scaffold the vulnerable epithelium.

To prepare the acceptor site we used a method based on the pathology of adhesion between the epidermis and dermis in patients affected by JEB. In the skin of patients with type XVII collagen deficiency the loss of adhesion is present at the level of the lamina lucida of the epidermal basement membrane. This enables "en bloc" removal of interfollicular epidermis with just an adhesive plaster. The plaster is placed on the skin and then, when removed, takes off the epidermis leaving naked lamina densa.

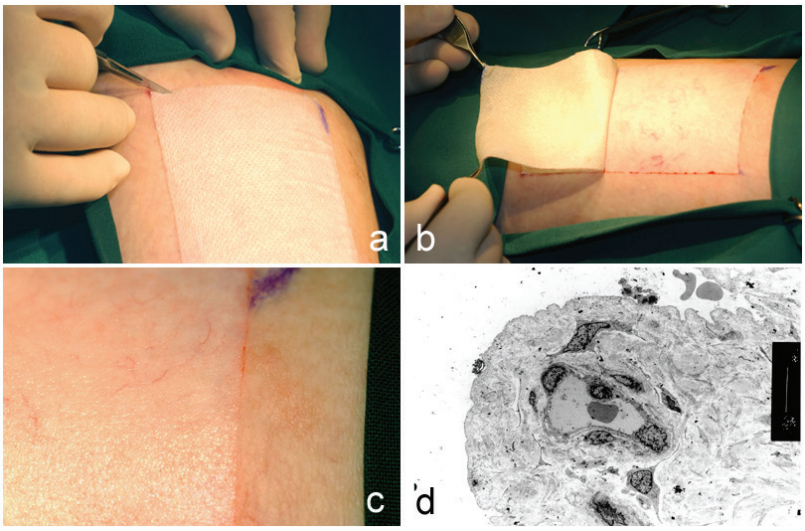

Figure 1. Preparation of the acceptor site on the upper leg. (a) Incisions are made around the adhesive plaster; (b) clear-cut peeling off of epidermis by adhesive plaster; (c) the bloodless wound bed is composed of naked lamina densa; (d) electron microscopy reveals complete removal of epidermis with the lamina densa on top of the wound surface.

Transplantation

The patient was hospitalized one day prior to transplantation. On the evening before the procedure, a 12x7 cm area was outlined on the right upper leg, degreased with ether, and a polyacrylate adhesive plaster (Fixomull stretch; BSN Medical Hamburg, Germany) was placed on the skin. The transplantation procedure took place under sterile conditions in the operating theatre. Paracetamol (500 mg) was given preoperatively. No local or systemic anaesthesia was used. During the transplantation ultrasuperficial incisions (<0,1 mm) were made around the edge of the plaster with a scalpel to direct the line of perforation of the epidermis. The plaster together with the attached epidermis was easily peeled off. A clean, nonbleeding erosion developed induced by a split at the level of the lamina lucida as demonstrated by electron microscopy of a skin biopsy, disclosing the naked lamina densa as the wound surface (Figure 1 a-d). The acceptor site was kept moist with warm saline gauzes and immediately before placing the graft it was carefully padded with dry gauze. The grafts were placed on the wound bed with the basal layer facing the wound.

As the first contact layer, polyurethane foam coated with silicon (Mepilex transfer, Mölnlycke Health Care, Breda, the Netherlands) was used, covered with nonadherent absorbent compresses (Melolin, Smith & Nephew). That was then fixed by a wrap of nonadhesive elastic gauze bandage (Elastomull Haft, Beiersdorf) and tubular stretch bandage (Tubifast Garment, Medeco). The patient was immobilized for three days and given nadroparin for thrombosis prophylaxis. [Figure 2 a-d]

Figure 2. (a) A revertant skin patch before harvesting with a punch biopsy (arrow, future donor site). (b) Epithelial grafts are placed on the acceptor site. Note the metal clips at the edge that secured the graft to the gauze carrier. (c) Removal of the gauze carrier at day 7. (d) The acceptor site had healed without scarring at 4 months. (e) Positive adhesive tape test of the acceptor site reveals weak epidermolysis bullosa skin. (f) The graft stained for type XVII collagen with 3% positive keratinocytes.

Results

The whole procedure was well tolerated by the patient. During the next five days dressings were left intact and no leakage of wound exudates was observed. Five days after transplantation all dressings, except the gauze scaffold were removed. The graft looked well attached to the acceptor site; the wound was dry. Seven days after the procedure all dressings, including the gauze, were removed. The wound was erythematous but dry and had re-epithelialized. No blisters were observed. Four months later the acceptor site had healed without scarring and with minimal

linear pigmentation. The adhesive pull test however provoked a skin loosening [Figure 2e]. Retrospective staining with monoclonal 1A8C (a kind gift from Dr K. Owaribe, Nagoya, Japan) for type XVII collagen of p2 cells side cultured on the cover slips showed that more than 30% of the cells were revertant. However the graft as the final product unexpectedly contained a low percentage of revertant cells (<3%) (Figure 2f). This explains why no functional repair was obtained, although the transplantation procedure was a surgical success.

Discussion

In our experiment we showed that the removal of epidermis with an adhesive plaster in patients with JEB is feasible. Our method was well tolerated, simple to perform and, very imporantly, causes little pain and therefore was not traumatic for the patient. After transplantation we have observed a fast and physiological healing process without any signs of graft rejection or scarring. That is why we suggest that the adhesive stripping could also be used for *ex-vivo* skin stem cell gene therapy in JEB which also requires replacement of large areas of epidermis.

The correction of the phenotype was unsuccessful because the graft consisted of less then 3 % revertant cells. There is no clear explanation why the graft appeared to be negative. The biopsy was taken from a patch of healthy skin, where at least 30 % of the cells were revertant. We have as yet no answer to this problem and any kind of discussion would be highly speculative. There is a possibility that during the wound healing process resident mutant epidermal stem cells colonize the grafted bed from the edge of the wound or from skin appendages. Theoretically it could be a danger for the revertant graft. However we have no reason to believe that these mutant cells would outcompete the grafted revertant cells. In previously described cases of transplantation of epidermal grafts,[2,4] graft survival was demonstrated. The method of acceptor site preparation by diathermy which left dermal papillae intact, was not deep enough to remove hair follicles, but was sufficient for long term graft survival in the first *ex-vivo* gene therapy performed in the skin.[2] We believe that the revertant cell therapy would be an elegant way to study the survival of autologous keratinocytes after epithelial sheet grafting.

Conclusion

We conclude that adhesive tape stripping is a simple, effective and almost painless method for acceptor site preparation in revertant cell therapy. The method might also work for grafting of genetically modified epithelial sheets in EB. The next hurdle in revertant cell therapy in EB is to improve graft production such that adequate percentages of revertant stem cells are present, to secure functional repair of the skin.

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3

LONG-TERM SURVIVAL OF TYPE XVII COLLAGEN REVERTANT CELLS IN AN ANIMAL MODEL OF REVERTANT CELL THERAPY

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Published in The Journal of Investigative Dermatology 2014 Feb;134(2):571-4

TO THE EDITOR

Revertant mosaicism (RM) is the coexistence of mutant cells carrying germline mutations and revertant cells that have spontaneously corrected the germline mutation by a somatic reverse mutation. Revertant mosaicism has been reported for a number of genetic diseases,[1] including epidermolysis bullosa. Moreover, the first case of revertant mosaicism in skin was found in a Dutch patient 026-01 with junctional epidermolysis bullosa caused by mutations in *COL17A1*. The patient was compound heterozygous for a maternal deletion in exon 18, c.1601delA, and paternal nonsense mutation in exon 51, c.3676C>T.[2] Due to gene conversion the c.1601delA mutation was corrected and the patient presented a clinically healthy skin patch on her forearm (Figure 1a), where affected (mutant) and corrected (revertant) keratinocytes co-existed (Figure 1b). Recently, we found revertant mosaicism to occur in all Dutch patients with junctional epidermolysis bullosa.[3,4]

Naturally corrected keratinocytes expressing type XVII collagen (Col17) harvested from a revertant patch can be used for autologous cell therapy. Unfortunately, the first attempt to transplant revertant keratinocytes did not succeed because of a surprisingly low percentage of revertant cells in the graft (3%).[5] To explain the depletion of revertant cells we have analyzed the process of skin equivalent production. Additionally, we have decided to study the feasibility of revertant cell therapy in an animal model to assess the long-term survival of revertant cells after engraftment. Written, informed consent was obtained, and all procedures were conducted in accordance with the Declaration of Helsinki principles.

Our revertant cell source was the revertant patch on the forearm of patient 026-01 (Figure 1a). A previous biopsy specimen from this area stained for Col17 showed 50% revertant cells (Figure 1b).[2,4,6] Next to the earlier diagnostic biopsy location a 6-mm punch biopsy was taken (Figure 1c) from which keratinocytes and fibroblasts were isolated with collagenase and trypsine/EDTA enzymatic digestion, and cultured as described before.[7] Keratinocytes were cultured on a feeder layer and passaged when confluent to expand the culture for animal model experiments and banking. The first passage was performed 9 days after isolation and the second passage (p2) was done 7 days later. During both passages a side sample was taken, cultured on coverslips, airfixed and the number of revertant cells was assessed by immunofluorescence (IF) microscopy with the 1A8C monoclonal antibody (mAb) against Col17 (gift from Dr. K. Owaribe, Nagoya, Japan). Stainings showed 40% revertant cells after first passage, and 25% revertant cells after the second passage (Figure 1e and 1i). A sample of the keratinocytes was further cultured, which led to a drop of revertant cells to 15%, 1% and <1% after passage 3, 4 and 5, respectively. Growth potential was tested with colony forming efficiency assay and showed that cells isolated from the revertant patch had growth potential comparable to keratinocytes from a healthy patient (Supplementary 1). Colonies were further stained with VK4 mAb, which revealed that colony

forming potential was higher in revertant cells (39% of colonies were revertant compared with 15% of revertant cells in the population used for the assay), but their division rate represented by colony size, was lower (Figure 1d).

From cultured patient's fibroblasts and p2 keratinocytes, a 75cm^2 bioengineered skin equivalent was produced as described before.[7,8] Briefly, a plasma-based scaffold filled with fibroblasts was used as a dermal component of the bioengineered revertant skin: 7.5x104 cultured fibroblasts were resuspended in 10 mL of donor plasma (obtained form the blood bank), 10 mg of tranexamic acid (Amchafibrin, Rotapharm, Barcelona, Spain), 2 mL of $CaCl_2$ 1% and adjusted to 25 mL by adding NaCl 0.9%. The mixture was placed in a 75 cm^2 tissue culture flask and allowed to solidify at 37°C for 30 minutes. Once the dermal equivalent solidified, it was covered with culture medium and 24 h later cultured keratinocytes were seeded. After 8 days, when keratinocytes reached confluence, the skin equivalent was harvested and moved to the animal facility. The equivalent was then divided into four pieces, 9cm^2 each, for grafting and samples were frozen for the Col17 immunofluorescence analysis. The sample sections were stained with 1A8C mAb against Col17 and analysed by measurement of Col17 positive fragments. This analysis revealed that 20% of the graft consisted of revertant cells (Figure 1f).

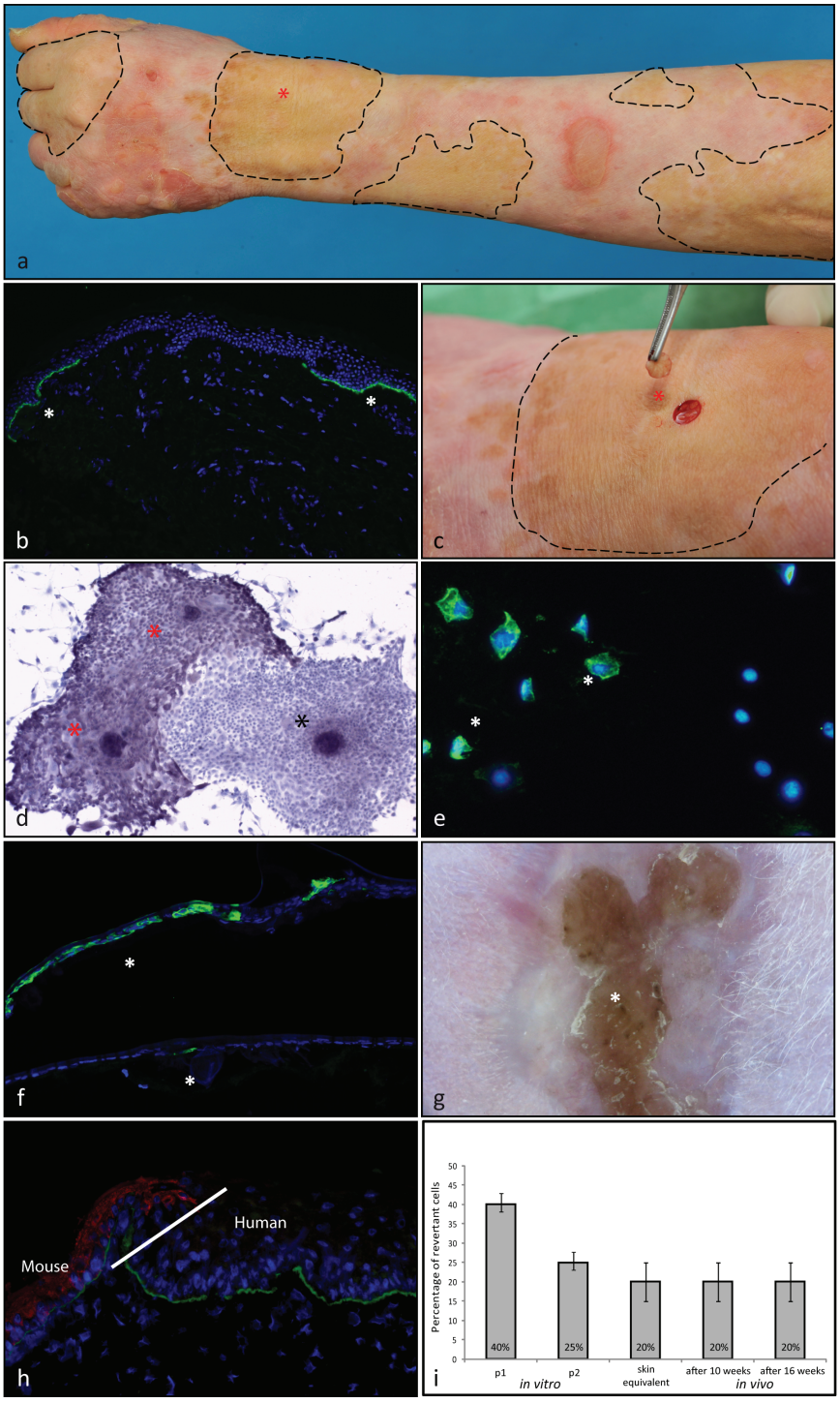
Four immunodeficient nu/nu mice were transplanted as described before.[8,9] Biopsies from the engrafted skin (Figure 1g) were taken after 10 and 16 weeks and directly frozen in liquid nitrogen. Each of the four biopsies was cut into 4-µm sections and stained with 1A8C or VK4 mAb (dr. H. H. Pas, Groningen, the Netherlands)[10] against Col17 to assess revertant cell survival, and with mAb (AF109) specific for murine keratin 1 (Prof. D. Roop, Denver, U.S.A.)[11] to distinguish human and murine epidermis. Once again, length of Col17 positive fragments and the total length was measured and analysis revealed that revertant cells were present in 20% of the human epidermis after both time points (Figure 1h and 1i). This corresponded with the percentage of revertant cells found in the skin equivalent and suggested a long-term survival of Col17 producing cells. To prove that the reversion mechanism was present in the areas positive for Col17, laser dissection microscopy (LDM) followed by nested PCR were performed on all biopsies as described earlier.[6] In fragments of human epidermis positive for Col17 we found the same postzygotic reversion mutation as in donor site keratinocytes on the patient's forearm, i.e. the loss by gene conversion of the maternal *COL17A1*: c.1601delA mutation.

The reason for a decreased percentage of revertant cells remains unknown. It has been shown that Col17 influences hair follicle stem cells in mice and that cells lacking Col17 have a different phenotype *in-vitro.*[12] Furthermore, Col17 deficiency activates a pro-inflammatory pathway by up-regulation of NF-κB, whereas in healthy keratinocytes that express Col17, levels of NF- κB are low.[13] Therefore, the growth advantage in cultures of Col17-negative keratinocytes over Col17-positive keratinocytes might be explained by NF-κB activation that stimulates epidermal

proliferation in the Col17 negative cells.[14] Further studies are required to assess whether such differences account for a growth advantage of mutant keratinocytes. To overcome the *in-vitro* depletion of revertant cells for a therapeutic approach, a method for selection of Col17 revertant cells compatible with clinical patient care is needed.

In this study we show that after a marked decrease during *in-vitro* expansion on plastic, the percentage of revertant keratinocytes stabilizes during skin equivalent production and remains stable *in-vivo* (Figure 1i). This proves long-term survival of revertant keratinocytes suggesting that we transplanted revertant epidermal stem cells. Hereby we demonstrate the feasibility of the revertant cell therapy for Col17 deficient patients.

Figure 1 (right). **(a)** Patient 026-01: left arm, outlined revertant patches, red asterisk marks the location of previous diagnostic biopsy; **(b)** Immunofluorescence of the diagnostic biopsy showing 50 % of the cells being reverant (white asterisk); **(c)** Biopsy for cell culture is taken from the revertant patch (outlined) next to the previous diagnostic biopsy (red asterisk); **(d)** Immunohistochemical staining of the colony forming efficiency assay with VK4 mAb showing two small revertant colonies (red asterisk) next to a large mutant colony (black asterisk); **(e)** Cultured keratinocytes on coverslips stained with 1A8C mAb (green) against the intracellular domain of Col17. In blue cell nuclei; **(f)** Samples of skin equivalent stained with 1A8C mAb (green). In blue cell nuclei. White asterisk indicates the revertant regions of the epidermis; **(g)** Revertant graft (asterisk) on mouse back 6 weeks after transplantation. Pigmentation is visible where human skin is present; **(h)** Graft on mouse sampled 10 weeks after transplantation and stained with VK4 mAb (green) against the intracellular domain of Col17 and with mAb against mouse keratin 1 (red). In blue cell nuclei. The white line separates murine (Mouse) and human (Human) epidermis; **(i)** Percentage of revertant cells during *in-vitro* culture, production of the skin equivalent and after transplantation. For *in-vitro* analysis after passage 1 and passage 2, 4761 and 2924 cells were counted, respectively.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Percentage of revertant cells
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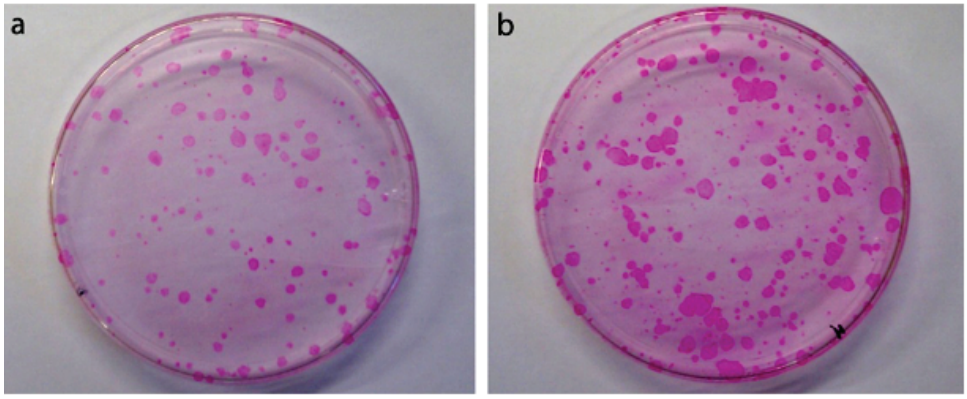
20%
20%
p1
p2
skin equivalent
after 10 weeks
after 16 weeks
in vitro
in vivo

SUPPLEMENTARY

Colony Forming Efficiency (CFE) assay of wild type keratinocytes isolated from normal human skin **(a)** and keratinocytes isolated from the revertant patch of patient 026-01 **(b)**. Cells were seeded for CFE at passage 3. Briefly, 1000 cells were seeded per 100mm dish and cultured on a feeder layer for 14 days, followed by fixation in 10% buffered formaldehyde and subsequent staining with a 1% Rhodamine B solution. Colonies larger than 2mm in diameter were counted. CFE values were 9,7 % ± 0,4 % and 10,4±2,6% (n=3) for wild type and patient's keratinocytes respectively.

Parallel colony assays were fixed with ice-cold acetone/methanol for 10 minutes and stained with VK4 monoclonal antibody against type XVII collagen to visualize revertant cells. The total number of non-abortive colonies was 204, which consisted of 80 revertant (39%) and 124 mutant (61%) colonies. The percentage of revertant colonies (39%) was higher than the percentage of revertant cells in the original population (assessed by staining of cover slips, 15%) suggesting higher potential of Col17-positive cells to form colonies when seeded sparse.

Mutant colonies, however, were on average bigger than revertant ones, as measured with Adobe Photoshop CS3 and analysed with Microsoft Excel – median size of Revertant colony was 949 pixels (n=50) and of Mutant colony 2439 pixels (n=50).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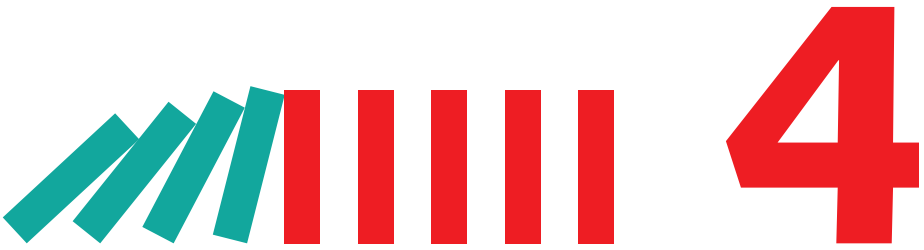

SUCCESSFUL THERAPEUTIC TRANSPLANTATION OF REVERTANT SKIN IN EPIDERMOLYSIS BULLOSA

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Published in The Journal of the American Academy of Dermatology 2014 Jan;70(1):98-101

ABSTRACT

Background. Epidermolysis bullosa (EB) is a group of genetic blistering diseases. Despite many efforts, treatment for EB remains symptomatic. Revertant mosaicism, coexistence of cells carrying disease-causing mutations with cells in which the inherited mutation is genetically corrected by a spontaneous genetic event (revertant cells) in one individual, can be found in EB. The naturally corrected revertant keratinocytes provide a unique opportunity for autologous cell therapy.
Objective. We sought to locally treat EB by transplantation of revertant skin.
Methods. Persistent ulcers in a patient with non-Herlitz junctional EB caused by to mutations in the *LAMB3* gene were treated by transplantation of split-thickness biopsy specimens from one of his revertant patches.
Results. All transplanted biopsy specimens were accepted and complete re-epithelialisation occurred within 14 days. During 18 months of follow-up, the patient never experienced blisters or wounds in the grafted area, nor in the healed donor site. Immunofluorescence and DNA sequencing showed that acceptor sites healed with transplanted revertant keratinocytes.
Limitations. Punch grafting allows only limited expansion of revertant skin.
Conclusions. We demonstrate that phenotypical and genotypical correction of skin in patients with revertant mosaicism by expansion of revertant skin might be a promising therapeutic option for cutaneous manifestations of EB.

CAPSULE SUMMARY

1. There is yet no curative therapy for EB available. Common occurrence of revertant mosaicism in patients with junctional EB holds therapeutic promise.
2. Autologous transplantation of revertant skin results in reversion of phenotype and genotype and requires no genetic manipulation.
3. Use of revertant patches as a therapeutic platform may become an easy and available method to treat the skin of patients with EB.

INTRODUCTION

Epidermolysis bullosa (EB) is a group of genetic blistering diseases caused by mutations in more than 17 different genes, including *LAMB3*. Patients with EB experience lifelong trauma induced blistering of the skin and mucous membranes together with extra-cutaneous symptoms. Despite many efforts, treatment for EB remains symptomatic and is mainly focused on wound care.[1] Skin grafting, in the form of dermal substitutes, cultured skin equivalents, or full-thickness and split-thickness autografts have been reported to treat wounds in patients with EB.[2-8] Recently our group has described autologous punch grafting of healthy looking but genetically affected skin to treat chronic wounds originating from persistent blisters in patients with the junctional form of EB.[9] With the healing rate of 70% this approach proved to be successful. However, all of the aforementioned methods for the treatment of EB wounds do not reverse the disease and therefore do not protect patients from recurrence of blisters and chronic wounds in the treated areas. The study conducted by Yuen et al. in our clinic showed a 16% recurrence rate in four patients.[9] However, as stated by the author, the follow-up time was relatively short with a median of 6 months and range of 1 to 25 months.[9] Moreover, extended unpublished follow-up of one of these patients resulted in an almost 100 % recurrence rate in his grafted areas. Thus there is a need for a curative treatment for patients with EB.

Revertant mosaicism, the coexistence of mutant cells carrying germline mutations and revertant cells that have spontaneously corrected the germline mutation by a somatic reverse mutation, is seen in genetic diseases of the skin, liver and blood.[10] In EB, revertant mosaicism has already been described in patients with mutations in the *COL17A1*, *COL7A1*, FERMT1, KRT14 and *LAMB3* genes.[11] The presence of spontaneously corrected revertant keratinocytes provides a unique opportunity for autologous cell therapy and for the generation of patient-specific induced pluripotent stem cells.[12, 13] We have previously described efforts to transplant cultured revertant skin grafts, which were unsuccessful.[14] Here we report a case of successful therapeutic transplantation of revertant skin leading to the reversion of disease in the treated region.

METHODS

Patient and intervention

Patient 029-01, a 69-year-old man with non-Herlitz junctional EB due to a homozygous splice-site mutation in the *LAMB3* gene (c.628G>A) had been confined to a wheelchair since 1993 after transfemoral left leg amputation as a result of cutaneus squamous cell carcinoma.[15] He presented with 7 persistent (>1 year) and painful ulcers: six on the lower aspect of his back (Figure 1b) and one on his right foot. The total area of the ulcers was 7 cm^2 and no signs of malignancy were present. On the patient's body multiple revertant skin patches have been

previously identified.[16] The large revertant area on the patient's right shoulder (Figure 1a) exhibited two different reversion mechanisms, c.628+3C>T and c.629-1G>A, both leading to an in-frame *LAMB3* transcript (Figure 1i). For treatment of his ulcers we chose to use punch biopsy specimens from this revertant skin patch on his right shoulder.

The revertant skin patch was anesthetized with a field block by local injections of lidocaine (20mg/ml)/epinephrine (5µg/ml) and 73 3-mm split-thickness punch biopsy specimens were harvested and placed on a wet gauze (Figure 1a). Simultaneously wound debridement in all ulcers was performed. Harvested biopsy specimens were then divided between all ulcers and positioned in the wounds (Figure 1c). A silicone exudate transfer dressing (Mepilex Transfer®) was applied and covered with silicone foam dressing (Mepilex®). The patient was advised strict bed rest for the first five days after the procedure and was hospitalized for a total of seven days.

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RESULTS

Inspection took place five and seven days after the procedure during which adequate wound healing and no signs of infection were observed. All 73 transplanted punch biopsy specimens were accepted in the grafted area and complete re-epithelialisation occurred within 14 days (Figure 1d and 1e). Interestingly, 21 days after the procedure a blister appeared next to one of the treated ulcers. The blister did not spread into the transplanted area (Figure 1d), suggesting that the healthy phenotype had been restored. During the course of 18 months healing and blister formation were assessed. The patient was seen a total of 14 times by a dermatologist with a maximal interval of 2 months. Parallel to these evaluations he was asked to self-asses transplanted areas and, if in doubt, send photographs. In this follow-up period the patient never experienced blisters or wounds in the grafted area, nor in the healed donor site. In addition, he reported an improvement in carrying out his daily activities and was completely relieved of pain in the treated areas.

Immunofluorescence staining for laminin-332 (lam-332) with GB3 monoclonal antibody on sections from both donor and acceptor sites after 10 weeks (Figure 1e, f, j and k) revealed levels of lam-332 expression comparable with normal-appearing control skin (Figure 1g), where as the biopsy specimen of mutant skin showed reduced intensity of lam-332 (Figure 1h). Furthermore, DNA analysis of revertant skin cells isolated from the donor and acceptor sections by laser dissection microscopy showed the presence of the same postzygotic reversion mutations in all samples (Figure 1i-1k), indicating that the donor site healed with revertant epidermis and that the acceptor sites were populated by the transplanted revertant keratinocytes.

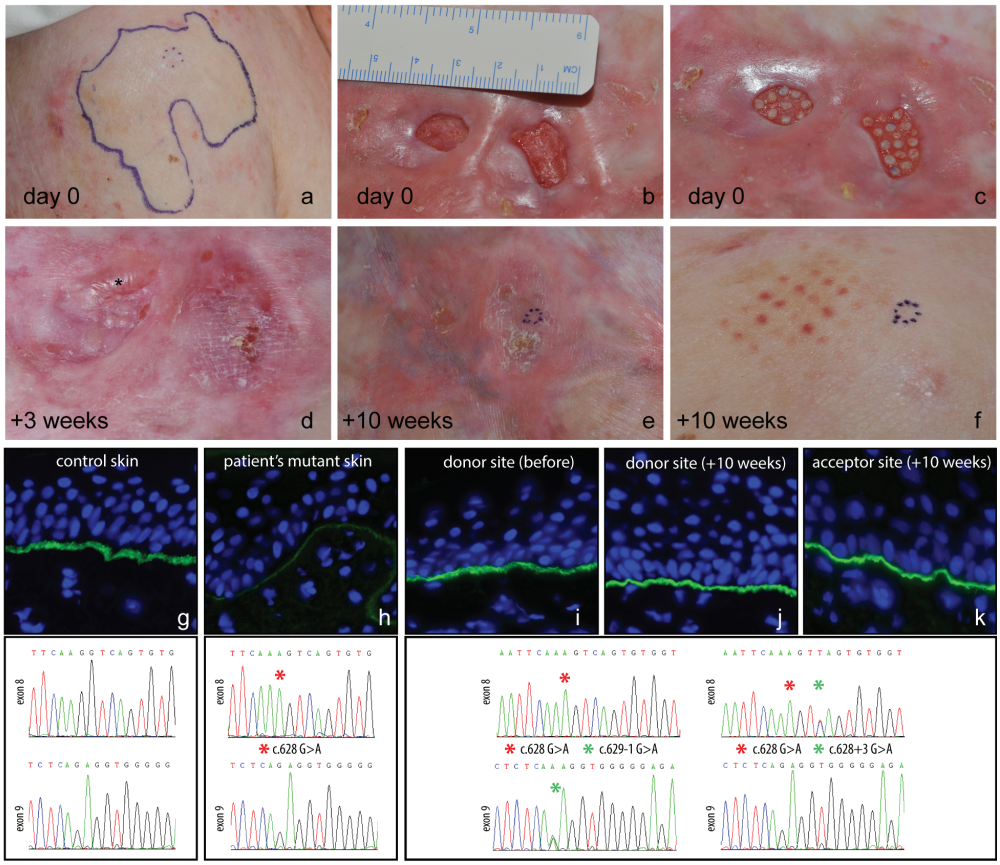

Figure 1. Junctional epidermolysis bullosa. Transplantation of revertant skin in a laminin-332 deficient patient. Transplantation of revertant skin: **(a)** outlined revertant patch (donor site) on patient's right shoulder and **(b-e)** acceptor site in patient's lumbal region: two ulcers before transplantation **(b)**, directly after placement of biopsy specimens **(c)**; healed ulcers after 3 weeks **(d)** and 10 weeks **(e)**. In **(d)** asterisk marks a blister that is not spreading to the transplanted region. In **(f)** the donor site 10 weeks following the procedure. Immunofluorescence staining for lam-332 (green) and DNA sequencing of LAMB3 **(g-k)**. Biopsy sites **(i)**, **(j)** and **(k)** are marked in **(a)**, **(f)** and **(e)** respectively. Normal **(g and i-k)** and reduced **(h)** levels of lam-332 are visible in basal membrane zone. Corresponding DNA sequences with marked the germline c.628G>A (red asterisk) mutation and somatic reverse mutations (green asterisk). In samples from donor and acceptor sites the same reverse mechanisms were found.

DISCUSSION

Transplantation of revertant skin, and therefore expansion of healthy, disease free skin, can highly improve quality of life in patients with EB. Earlier attempts, focused on cultured skin equivalents, were not successful because of an insufficient amount of revertant cells in the grafts (<3%).[14] In this report we show that both donor and acceptor sites healed with revertant epidermis and expressed amounts of lam-332 comparable with normal-appearing human skin. Moreover, by tracing the reversion mechanism from the donor site on the shoulder, we know that the keratinocytes found in the healed ulcers originated from the donor revertant patch. This proves that our intervention led to expansion of the revertant surface area of the skin.

In comparison with earlier reported methods for wound treatment in EB, our intervention resulted not only in healing of all treated wounds, but also restoration of a healthy phenotype and genotype. In autologous transplantation of mutant skin, one can expect blisters and chronic wounds in the treated area (donor site) because the graft carries the inherited mutation as seen in two patients described by Yuen et al.[9] Our findings show that even 18 months after revertant skin is transplanted blisters do not spread into the treated area and the treatment is not only symptomatic but also curative.

The advantage of using a punch graft for the treatment of chronic wounds in EB with revertant skin is the small size of each single graft. This is of great importance because revertant patches on the patient's body are often small, multiple in number and irregular in shape and therefore the small size of punch biopsy specimens gives better control over which area is harvested and maximizes their use.

Stable and long-lasting reversion of the EB phenotype by transplantation of revertant skin in a patient has a big impact on the development of revertant cell therapy. Treatment of the whole body surface will definitely be possible by application of cultured epidermal revertant grafts. However, if revertant cell therapy is to treat larger areas of mutant skin in one patient, methods allowing for higher *in-vitro* expansion of revertant keratinocytes are needed.

In conclusion, we show that phenotypical and genotypical correction of skin in patients with EB by transplantation of revertant skin is a promising therapeutic option for cutaneous manifestations of EB.

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5

PIGMENTATION AND MELANOCYTE SUPPLY TO THE EPIDERMIS DEPEND ON TYPE XVII COLLAGEN

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Published in Experimental Dermatology 2014 Feb;23(2):130-2

ABSTRACT

Genetic deficiency of type XVII collagen (Col17), laminin-332, or type VII collagen causes epidermolysis bullosa (EB). Spontaneous correction of the deficiency, also known as revertant mosaicism, is caused by a second somatic mutation that restores protein expression resulting in clinically healthy (revertant) patches surrounded by fragile (mutant) skin. Interestingly, in some patients patches of revertant skin show hyperpigmentation. To study the possible role of affected proteins in pigmentation and melanocyte distribution, we investigated clinical documentation and skin biopsy specimens of 13 revertant EB patients having correcting mutations in the *COL17A1*, *LAMB3*, or *COL7A1* genes.

Analysis revealed that lack of Col17 led to decreased melanin intensity and melanocyte density in the epidermis when compared to the revertant patches. Reversions of *LAMB3* and *COL7A1* in keratinocytes did not influence clinical pigmentation or density of melanocytes. We conclude that in human skin, melanocyte supply to the epidermis depends on Col17 expression in keratinocytes.

BACKGROUND

Melanocytes are known as melanin producing cells in the epidermis and hold other functions, for example neuroendocrine in the brain and metabolic on retinoids in the eye.[1] In the skin, melanocytes are derived from the neural crest during embryonic development. In the interfollicular epidemis (IFE) melanocytes are not attached by hemidesmosomes, but by poorly defined dense plaques.[2] Resting melanocytes do not synthesize adhesion proteins such as type XVII collagen (Col17), laminin-332 (lam-332) or type VII collagen (Col7).[2,3]

Col17, lam-332 and Col7, along with 11 other macroproteins, are synthesized by keratinocytes and involved in the group of genetic blistering diseases called epidermolysis bullosa (EB).[7-9] Revertant mosaicism (RM) refers to the coexistence of cells carrying the original germline mutation and cells that spontaneously have corrected the germline mutation by a somatic reverse mutation in one individual[4]. This reverse mutation leads to a clinically healthy phenotype. In the last 15 years RM has been identified for five EB genes – *COL17A1*, *LAMB3*, *COL7A1*, *FERMT1*, and *KRT14*.[11-14] Reversion in EB has only been observed in keratinocytes and not in fibroblasts, melanocytes or peripheral blood cells.[5,6] The first patient with RM in EB (patient 026-01) described by Jonkman et al. had a generalized subtype of non-Herlitz junctional EB (JEB-nH-gen) due to *COL17A1* mutations.[7] She had a revertant (healthy) patch on her hand due to a gene conversion. This patch was more pigmented than the surrounding, mutant (affected) skin. Interestingly, revertant skin manifested as hyperpigmented patches in several patients.[8,9]

QUESTIONS ADDRESSED

The spontaneous coexistence of two different microenvironments, that is mutant and revertant epidermis, within one individual may help to answer, if pigmentation and melanocyte biology in human skin depend on a protein of the epidermal basement membrane zone. We have performed a correlational study in a population of EB patients with RM for *COL17A1*, *LAMB3* and *COL7A1* and based on our results and current literature formulated a hypothesis about importance of Col17 for melanocyte supply.

EXPERIMENTAL DESIGN

We identified 13 patients with RM in the Dutch and Spanish EB databases: ten with JEB-nH-gen due to mutations in *COL17A1* (n=8) or *LAMB3* (n=2), and three with recessive dystrophic epidermolysis bullosa (RDEB) due to mutations in *COL7A1* (Table 1). As a control group we selected six biopsies of healthy Caucasian individuals (Table 1). Furthermore, four localized JEB-nH patients (JEB-nH-loc) were included, because of their intermediate level of Col17 expression.[10]

Clinical phenotype and pigmentation of mutant and revertant skin were assessed. Additionally, biopsies of all patients and control individuals were stained for Col17, lam-332, Col7, melanocytes and melanin (Supplementary).

RESULTS

In the *COL17A1* revertant patient group all patients showed hyperpigmentation of revertant skin (Table 1). The patches were sharply demarcated by pigmentation with characteristics of healthy skin with normal skin texture (Figure 1A). Dermatoscopy aided clinical differentiation between revertant skin and EB naevi (Figure S1A-C). In contrast, revertant patches in both *LAMB3* and *COL7A1* patients showed no clear difference in pigmentation. Their revertant skin could be distinguished from the surrounding affected skin by lack of erythema (Figure 1B and 1C). Fontana-Masson staining for pigment corrals (Figure 1E and S1E) in *COL17A1* revertant patients showed more pigment present in the revertant patches than in the mutant skin (Figure S1G). Such a trend was not observed in the two other groups (Supplementary). Immunofluorescence staining revealed a lower density of melanocytes in the mutant skin (median: 2.60 melanocytes/1000µm basement membrane; Figure 1D, 1F, 1G, S1D and S1F), when compared with revertant skin (median: 9.41) of *COL17A1* revertant patients. Surprisingly, *LAMB3* revertant patients showed a lower density of melanocytes than the control group, yet no difference was found between revertant (median: 4.38) and mutant (median: 4.23) skin (Supplementary). Taken together, pigmentation and melanocyte density were significantly lowered only in Col17 deficient JEB-nH-gen skin.

DISCUSSION

Col17 is a transmembrane hemidesmosomal protein synthesized in the skin by keratinocytes.[11,12] This study demonstrates that lack of Col17 significantly impairs melanisation and decreases the number of melanocytes in skin ($p<0.01$), whereas restoration of Col17 expression in revertant skin results in normal melanisation and melanocyte density. Our work confirms that revertant patches in Col17 deficient patients can clinically be recognized by hyperpigmentation. Interestingly, we found normal melanocyte density in the skin of JEB-nH-loc patients meaning that slightly reduced Col17 levels are sufficient for normal melanocyte distribution. Therefore, the amount of Col17 needed for maintaining the melanocyte population seems to be lower than that needed for maintaining a healthy dermal-epidermal junction.

Recent findings of the important role of Col17 in melanocyte stem cell survival, cell signalling and immune response might explain loss of melanocytes in Col17 negative skin. Nishie et al.[13]

and Tanimura et al.[3] showed hair greying and alopecia in Col17 -/- mice due to hair follicleatrophy. Tanimura et al. explained how hair follicle stem cells (HFSCs) and melanocyte stem cells (MelSCs) interact with each other in murine hair follicles.[3] Lack of Col17 expression results in reduced paracrine stimulation of MelSCs with TGF-β and leads to follicle atrophy due to depletion of both HFSCs and MelSCs.[6,23,24] As universal alopecia is also characteristic in completely Col17-deficient JEB-nH-gen patients, the same mechanism might be responsible for melanocyte depletion in Col17 mutant skin. The recent work of Van den Bergh et al.[11] showed that Col17 is involved in the immune response. Col17 negative keratinocytes had much higher levels of NF-κB than Col17 positive cells. This leads to overstimulation and an increased IL-8 response, which can attract T-cells and promote autoimmune targeting of melanocytes. IL-8 together with IL-6 can also directly inhibit melanocyte growth and modulate their antigen expression.[14-16]

All studied patients with *LAMB3* mutations had a lower than normal density of melanocytes and no melanin in both revertant and mutant skin. Reversion of keratinocytes in healthy patches did not rescue the melanocytes, as we saw in the *COL17A1* revertant group. Lam-332, in contrast to Col17, can be synthesized and secreted by actively dividing melanocytes and it has been earlier implied that lam-332 might be important for melanocyte proliferation.[2] This could possibly explain the lower density of melanocytes in patients with *LAMB3* mutations, as melanocytes are not revertant and thus not able to express lam-332 in *LAMB3* revertant and mutant skin. Further, our results suggest that Col7 is not directly involved in melanocyte biology as melanocyte distribution and pigmentation in both revertant and mutant *COL7A1* skin were normal.

In conclusion, we have proved that hyperpigmentation is a sign of RM in patients with mutations in *COL17A1*. Moreover, we show that there is a correlation between the levels of Col17 expression and presence of melanocytes in human epidermis, which deem further studies necessary to understand the underlying mechanism.

Phenotypic differences										
Affected gene	Clinical diagnosis	Patient	Difference in pigmentation between mutant and revertant skin	Hair growth		IF of the mutant skin				Reference
				Scalp hair	Body hair	Type XVII Collagen		Laminin-332	Type VII collagen	
						IC (1A8C)	EC (Lu-226/233)			
COL17A1	JEB-nH-gen	025-01	Yes	Absent	Absent[1]	-	-/+	+	+	9,17
		026-01	Yes	Absent	Absent	-	-/+	+	+	8,10
		035-01	Yes	Absent	Absent	-	-/+	+	+	
		035-02	Yes	Sparse	Absent[1]	-	+	+	+	
		093-01	Yes	Sparse	Absent	-	+	+	+	
		134-01	Yes	Normal	Absent[1]	-	+	+	+	
		208-01	Yes	Normal	Absent	-	+	+	+	8
		248-01	Yes	Absent	Absent	-	ND	+	+	in preparation
	JEB-nH-loc	086-01	N/A	Normal	Normal	-/+	+	+	+	10
		098-01	N/A	Normal	Reduced	-/+	+	+	+	
		168-01	N/A	Normal	Normal	-/+	+	+	+	
		224-01	N/A	Normal	Normal	ND	+	+	+	
LAMB3	JEB-nH-gen	029-01	No	Normal	Reduced	+	+	-/+	+	5
		078-01	No	Normal	Reduced	+	+	-/+	+	
COL7A1	RDEB	024-01	No	Normal	Normal	+	+	+	-/+	18
		260-01	No	Normal	Absent	+	+	+	-/+	19
		265-01	No	Normal	Absent	+	+	+	-	in preparation

Table 1: Clinical phenotype of included patients related to if antigen mapping of their mutant skin

Table legend: N/A – not applicable; ND – not determined; - negative; -/+ reduced; + positive; IC – intracellular domain of Col17; EC – extracellular domain of Col17; 1 single body hair growth observed on revertant patches.

SUPPLEMENTARY

Material and methods

Patients

We identified 13 EB patients with RM in the Dutch and Spanish EB registry database: ten with JEB-nH- gen *COL17A1* (n=8) and *LAMB3* (n=2), and three with recessive dystrophic epidermolysis bullosa (RDEB) due to mutations in *COL7A1*. As a control group we selected six biopsies of healthy Caucasian individuals, three taken from sun exposed skin and three from non-exposed skin. Furthermore, four localized JEB-nH patients (JEB-nH-loc) were selected, because of their reduced, but not absent, intermediate level of Col17 expression (10). Clinical phenotype and pigmentation assessments were based on information from EB- patient database, clinical photography and previous publications. The 4-6 mm diameter punch biopsies, taken previously from mutant and revertant skin of EB patients (5,7-10,19), mutant skin of JEB-nH-loc patients and healthy skin of control patients, were re-sectioned, stained and examined for this study. All biopsies used had been stored at -80C. Informed consent and approval of the local ethical committee was in accordance with the Declaration of Helsinki Principles.

Analysis of skin sections

We used the following monoclonal antibodies (mAbs) against Col17: 1A8C to the intracellular domain, 1D1 and 233 mAbs to the extracellular domain (all gifts from Dr. K. Owaribe, Nagoya, Japan)[20] for diagnostic assesment (Table 1) and VK5 to the intracellular domain (dr. H. H. Pas, Groningen, the Netherlands) for double staining with melanA; against lam-332: GB3 (Abcam) to γ2 chain; against Col7: LH7.2 (gift from I. Leigh, London, United Kingdom), and against the melanocyte marker melan-A: M2-7C10. Sections of 4-μm thickness were cut and air-fixed. VK5, LH7.2 and GB3 were conjugated with Zenon® Mouse IgG Labeling Kits AlexaFluor® 488 (green) and M2-7C10 with AlexaFluor® 568. mAbs were diluted in PBS/OVA 1% and sections were incubated at room temperature for 45 min. Hoechst 33342 was used to stain nuclei.
In each section melan-A positive melanocytes were counted under 63x magnification, and expression of proteins was assessed by optical comparison with control sections. The basal cell layer in all sections was photographed along the complete length of the specimen, and pictures of sectors were merged with Adobe Photoshop CS3 software. The length of the basal membrane was determined with image- processing software - ImageJ, version 1.44 (http://rsbweb.nih.gov/ij/index.html). The sections used for IF antigen mapping were incubated in PBS for 30 min to remove the cover glass, dried, and stained with Fontana-Masson to reveal melanin in the same section. Stained sections of each patient were scored semi-quantitatively by an experienced pathologist (section identity was blinded): 0 – no melanin, 3 – maximal amount of melanin, 0.5 step. The binominal test with p=0.5 was used to test statistical significance.

Results

Dermoscopy of Col17 revertant skin

In addition to the aforementioned results, in *COL17A1* revertant patients dermoscopic examination could be used to easily distinguish revertant skin (Figure S1A-C). Mutant skin presents a homogenous image with dotted vessels and no pigment network (Figure S1A), while revertant skin was identifiable by pseudo-pigment network with follicular pigmentation (Figure S1B). Patients 134-01 and 025-01 also had EB naevi, which can present different patterns, i.e. globular or cobblestone pattern as in case of these two patients (Figure S1C).[21]

Pigment staining

Differences in intensity of melanin between revertant and mutant skin were compared for each patient separately (Figure S1G). In the *COL17A1* mosaic group significantly more pigment was seen in all of the tested revertant skin than in their corresponding mutant skin (positive values in Figure S1G). Interestingly, in revertant biopsies where co-existence of revertant and mutant basal keratinocytes in one skin section could be seen, pigment allocation did not co-distribute with restored protein expression (Figure S1D-E). In the *COL7A1* revertant group, no correlation could be discerned between revertant and mutant skin. In all sections of *LAMB3* patients melanin was absent.

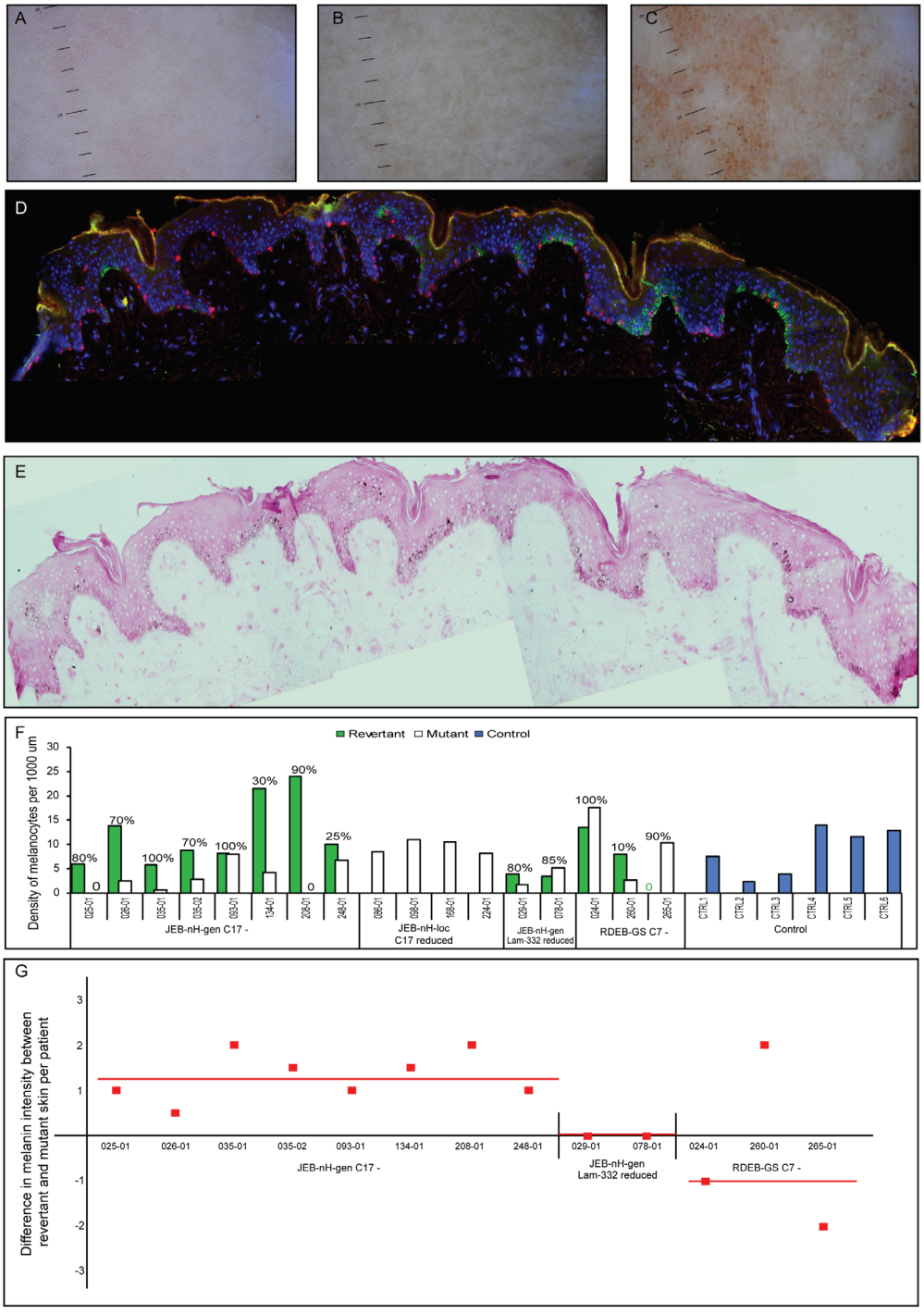
Melanocyte density

Median density of melanocytes in control group was 9.60 per 1000 µm of basement membrane, which corresponded to the melanocyte-keratinocyte ratio of 1/10.4. Patients mosaic for *COL17A1* presented with a density of melanocytes in their revertant skin (median: 9.41, ratio: 1/10.5) almost identical to our control group. Melanocytes were distributed evenly in IFE of each section and did not co-distribute with micro-mosaicism for *COL17A1* (Figure 1g and S1d). Their mutant, Col17 deficient, skin showed however a significantly lower melanocyte density (median: 2.60, ratio: 1/38.5). The four patients with JEB-nH-loc showed very similar results as control and *COL17A1* revertant group (median: 9.56, ratio: 1/10.5). *COL7A1* revertant patients had a normal number of melanocytes in mutant skin (median: 10.38, ratio: 1/9.5), and this number was slightly lower in revertant skin (median: 8.05, ratio: 1/12.5), but still within the normal values.

Micro-mosaicism

Skin sections of revertant skin often show micro-mosaicism, a coexistence of short segments of revertant and mutant keratinocytes within one slice, which we observed in 10 of 13 revertant biopsies (Figure 1D, S1D and S1F). When considering the influence of Col17 on melanocytes, we expected that in *COL17A1* revertant skin sections melanocytes would co-localize with revertant segments. However, melanocytes in these sections were evenly distributed throughout both the revertant and mutant segments. This might suggest a paracrine effect of Col17 on melanocytes in the human epidermis.

Figure S1. Dermoscopy of patients 134-01: a) mutant skin on the right wrist, b) revertant skin on the left arm and c) EB naevus. In mutant skin hypopigmentation and dotted vessels are seen, in the revertant skin a pseudo- pigment network and follicular pigmentation are clearly visible. EB naevus shows globular pattern with irregular dots. d) IF antigen mapping of Col17 in green, melan-A in red, and nuclei in blue. e) Identical section re-stained with Fontana-Masson (dark pigment grains). f) Melanocyte density (Y-axis: melanocytes/1000 µm BMZ) per patient, number above green bars shows the percentage of revertant cells (micro-mosaicism) within the biopsy. Green depicts revertant patients, white depicts mutant patients and blue depicts controls. g) Difference in melanin intensity (Y-axis, -3 to 3 value) per patient. Each square represents difference between revertant and mutant skin, positive result represents more pigment in revertant patch, while negative result represents more pigment in mutant patch. Red lines show median values per patient group.


A
B
C
D
E
F
Revertant
Mutant
Control
Density of melanocytes per 1000 um
80%
0
70%
100%
70%
100%
30%
90%
0
25%
80%
85%
100%
10%
90%
0
025-01
026-01
035-01
035-02
093-01
134-01
208-01
248-01
086-01
098-01
186-01
224-01
029-01
078-01
024-01
260-01
265-01
CTRL1
CTRL2
CTRL3
CTRL4
CTRL5
CTRL6
JEB-nH-gen C17 -
JEB-nH-loc C17 reduced
JEB-nH-gen Lam-332 reduced
RDEB-GS C7 -
Control
G
Difference in melanin intensity between revertant and mutant skin per patient
3
2
1
-1
-2
-3
025-01
026-01
035-01
035-02
093-01
134-01
208-01
248-01
029-01
078-01
024-01
260-01
265-01
JEB-nH-gen C17 -
JEB-nH-gen Lam-332 reduced
RDEB-GS C7 -

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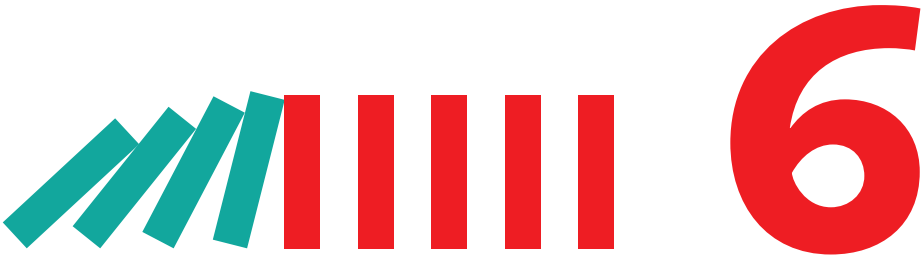

NOT ALL ROADS LEAD TO SUCCESSFUL REVERTANT CELL THERAPY

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In **Chapters 2-5** of this thesis we presented published work on the understanding of revertant mosaicism. Research on the development of revertant cell therapy in the Center of Blistering Diseases in Groningen that we have performed has been much more extensive than the experiments presented in **Chapters 2-5**. Throughout the process, many hypotheses and possible improvements to expand the revertant area of the skin were investigated. In this chapter I would like to present three selected experiments that were perhaps not a direct step forward on the path to revertant cell therapy but are worth mentioning, as they might have been the beginning of a new approach.

6

ENRICHMENT OF IN VITRO CULTURE WITH TYPE XVII COLLAGEN REVERTANT CELLS

We would like to acknowledge the participation of **Miranda Nijenhuis** and **Dr. Hendri Pas** in this study, both from the Department of Dermatology, University Medical Center Groningen.

Revertant cell therapy based on *in vitro* cultured skin grafts in patients with mutations in the *COL17A1* gene could profit from a technique that allows enrichment of revertant keratinocytes. *In vitro* enrichment could lead to a skin transplant with a higher percentage of revertant cells than those achieved in **Chapters 2 and 3**. A method for selection of revertant keratinocytes after isolation from the biopsy would ensure that the revertant cell population had chance to expand. Starting off, we tested different culturing methods, such as 3T3-J2 feeder layer conditions as described by Howard and Green and different defined, serum free media, such as defined-SFM keratinocyte culture medium (Gibco) or CnT-07 and CnT-57 (Cellntec). However, in all cases the percentage of mutant cells relative to the revertant cells increased with each passage as shown in **Chapter 3**.

Van den Bergh et al. described an interesting method to test cell attachment mediated by type XVII collagen (Col17) and laminin-332 (lam-332) with a method reported earlier by Reyes and Garcia.[1,2] In this method, a culturing plate is turned upside down and placed in the centrifuge. Results showed that cells, which do not express Col17, did not attach to the lam-332 coating as strongly as the Col17 expressing cells did. For example, when 55g force was used, 30 - 40% of Col17 expressing cells stayed attached to the plastic, while only 5% of Col17 negative cells was left in the culture wells. The cells tested by Van den Bergh et al, K562 and SK-MEL1, had a very low level of adherence on their own and the Col17 expression and attachment to lam-332 was their strongest attachment mechanism. We attempted to apply this technique to a mixture of *COL17A1* revertant and mutant keratinocytes, isolated from a biopsy of the patient described in **Chapters 2 and 3**. Unfortunately, primary keratinocytes do express many other adhesion molecules and therefore both populations remained strongly attached to different coatings, such as lam-332, type I collagen or even uncoated plastic when the protocol of Van den Bergh was followed. Time given for keratinocytes was shorten and under 5 minutes detachment of all cells was observed, while with longer times no difference before and after application of different g-forces was shown, up to 70g.

Another method to select revertant keratinocytes tested in our laboratory was sorting by flow cytometry. Col17 has an extracellular domain and therefore it is possible to stain living cells, after non-enzymatic detachment from the culture flask, with an antibody against the extracellular domain, such as the 233 monoclonal antibody (Gift from Dr. K. Owaribe, Nagoya, Japan).[3] First a staining protocol that allowed the removal of keratinocytes from the culture flask without

enzymatic digestion was established. Briefly, keratinocytes were incubated in a PBS/EDTA (Gibco) solution for 30 minutes and then cells were gently washed from the culture flask. Cells were then placed on ice in the DMEM/10%FCS (Gibco and HyClone, respectively) for 20 minutes. This method of detachment preserved the extracellular domain of Col17. We then established a staining method to label Col17 using the 233 monoclonal antibody with Alexa488-conjugated goat-anti-mouse IgG antibody as a second step (Invitrogen, USA). Cells were first incubated with the 233 monoclonal antibody that recognizes the extracellular domain of Col17 (1118-1143) for 30 minutes on ice and washed twice in PBS. This step was followed by incubation with the Alexa488-conjugated second step antibody for another 30 minutes and two additional washing steps at the end.

The staining protocol was first tested on healthy human keratinocytes and assessed by immunofluorescence microscopy. Green fluorescence indicating Col17 expression could be observed on the cell surface in both, the living cells and air-fixed cells samples stained with the 233 monoclonal (Figure 1 A and B). As a control antibody we used another Col17 monoclonal antibody VK4 (Dr. H.H. Pas, Groningen, the Netherlands), which is directed against an intracellular epitope of Col17. The sample of living cells stained with VK4 showed no fluorescent signal (Figure 1E), while the air fixed one did (Figure 1D). This means that the cell membrane remained stable in the living cells sample during the staining protocol because the VK4 was not able to penetrate the membrane and stain the intracellular epitope of Col17.

Next, keratinocytes were isolated from a revertant biopsy taken from patient EB026-01, described already in **Chapters 2 and 3**. Side samples of the culture showed 40% of revertant cells after the first passage and 18% after the second passage. For sorting with flow cytometer the same staining protocol was used as for the control cells and living cells were stained (Figure 1 F). Separated cells were further cultured under the same conditions as were used before cell sorting (CnT-07 culture medium, Cellntec).

Sorting of revertant and mutant cells with the flow cytometer facilitates further studies on revertant mosaicism. Enrichment of revertant keratinocytes by flow cytometry using monoclonal antibodies against Col17 is currently not allowed in the process of revertant graft production under good manufacturing practice (GMP). Possibly, different sorting techniques such as magnetic activated cell sorting (MACS) based on magnetic nanoparticles coated with antibodies could be easier certified for GMP conditions.

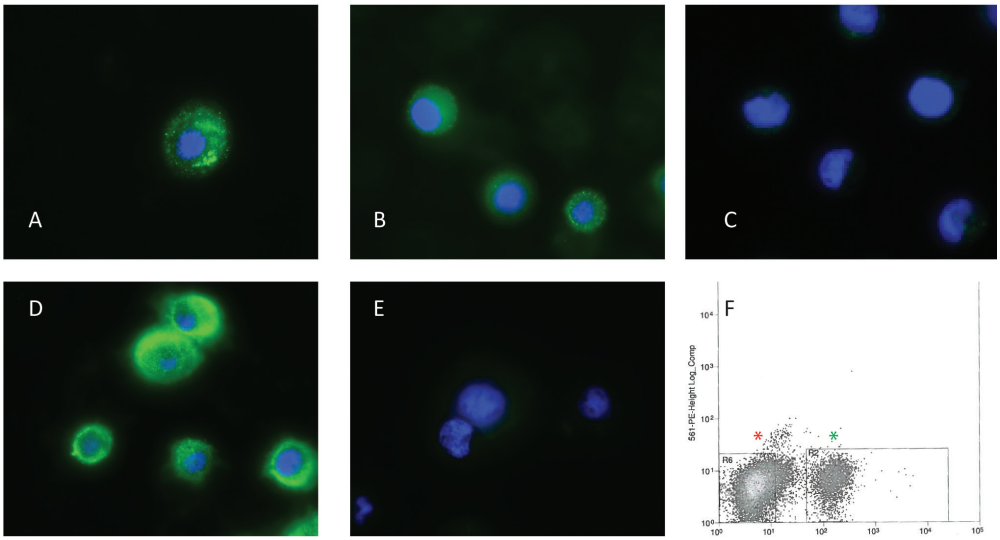

Figure 1. Immunofluorescence staining for sorting of Col17 positive cells with flow cytometer.

A and **D**: Air fixed wild-type keratinocytes stained with: **A.** 233 monoclonal antibody and **D.** VK4 monoclonal antibody. Col 17 is stained in both samples. **B** and **D:** living cells after non-enzymatic detachment, without fixation stained with **B.** 233 monoclonal antibody and **E.** VK4 monoclonal antibody. Only 233 antibody stains the cells meaning that the cell membrane remained intact. **C:** Cells after detachment with trypsin stained with 233 monoclonal showed no signal from the cell membrane. This suggested that the extracellular domain of Col17 has been cut off by trypsin. **F:** Sorting of revertant and mutant cells from patient EB 026-01 after passage 2. 77% of cells were labelled as mutant (R6) and 15% were labelled as revertant (R2).

REVERTANT CELL THERAPY IN A TYPE VII COLLAGEN REVERTANT PATIENT

This study was performed in collaboration with **Dr. Marta Garcia**, **Prof. dr. Marcela Del Rio** and **Dr. Fernando Larcher** from Regenerative Medicine Unit, CIEMAT and Centro de Investigaciones Biomédicas en Red de Enfermedades Raras (CIBERER U714) Madrid, Spain; and **Dr. Sara Llames** and **Dr. Alvaro Meana** from Tissue Engineering Laboratory, CCST-PA and Centro de Investigaciones Biomédicas en Red de Enfermedades Raras (CIBERER U714) Oviedo, Spain

In **Chapter 3** we described isolation of cells from a revertant skin biopsy of a Col17 deficient patient, production of skin equivalents and regeneration of human skin on the back of immunodeficient mice. When the first type VII collagen (Col7) revertant patients were identified in 2010,[4,5] it was decided to replicate the experiments described in **Chapter 3**, using the same murine model. This was done in order to test the feasibility of Col7 revertant cell therapy. Patient EB 260-01 was a female, homozygous for a single base pair insertion (c.6527dupC) in the *COL7A1* gene and therefore affected by recessive dystrophic EB (RDEB).[5] A revertant skin patch was observed on her left arm, where her skin never blistered (Figure 2A). Biopsies from this revertant patch showed levels of Col7 expression similar to the ones seen in the control biopsy and the reversion mechanism was identified by laser dissection microscopy to be a second-site mutation (c.6528delT).[5] In the biopsy described in the publication of Pasmooij et al. 85% of the biopsy area was revertant.[5]

A skin biopsy from the same revertant patch was taken and keratinocytes and fibroblasts were isolated. Cells were expanded in vitro to full thickness skin equivalents with the same technique that was used in the experiments described in **Chapter 3** and by Llames et al.[6] Mature skin equivalents were transplanted onto the back of nu/nu immunodeficient mice to obtain reconstituted human skin.[7,8] Side samples of cultured keratinocytes were analysed for the presence of Col7 revertant cells with immunofluorescence staining with LH 7.2 monoclonal antibody against the NC-1 terminus of Col7. Unfortunately, the percentage of revertant cells in those side samples was lower than 10% and therefore much lower than our expectations based on the biopsy described in 2010. Transplanted grafts after 10 and 16 weeks were analysed for possible changes in the revertant cell population during the *in vivo* phase of this experiment. Grafts were biopsied and stained with the monoclonal antibody LH 7.2 and a 234192 polyclonal antibody Calbiochem against Col7. Stainings revealed that in parts of the graft, some expression of Col7 was present (Figure 2B). The levels of expression in those parts were substantially lower than in the control graft (Figure 2C). We analysed the sections for presence of the reversion mechanism by first dissecting the fragments with the laser dissection microscopy and then sequencing the DNA in order to asses the presence of the correcting mechanism (c.6528delT). Only the original

homozygous c.6527dupC mutation was identified and the reversion mechanism c.6528delT was not present in the analysed sections.

We concluded that no revertant keratinocytes could be detected in the transplanted graft, because of the low percentage of the revertant keratinocytes in the original cell culture. Col7 seen in the analysed graft might be present as a result of upregulation of the mutant and thus non-functional Col7 expression. Changes in the dermal and/or epidermal homeostasis could result in the changes of Col7 expression in RDEB patients, as it was shown in trials using fibroblast injections.[9,10] An alternative explanation is the observation that a low level of Col7 expression has been observed in RDEB patients, due to a low level of exon skipping.[5] In the presented study we were not able to isolate and culture a sufficient number of Col7 revertant keratinocytes. We expect, however, that if a biopsy with a satisfactory percentage of revertant keratinocytes is obtained and cells are successfully isolated, a Col7 revertant skin graft could be obtained. This has to be, however, further investigated.

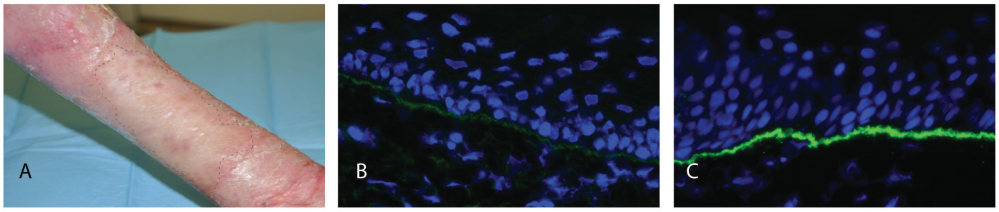

Figure 2. Murine model of Col7 revertant cell therapy. Immunofluorescence staining for Col7 of human grafts 10 weeks after transplantation on mice. **A.** Revertant patch on patient's arm. **B.** Graft 10 weeks after transplantation on the immunodeficient mice, stained with LH 7.2 monoclonal antibody. Depicted in green Col7 and in blue nuclei. **C.** Wild-type graft (10 weeks) stained with LH 7.2 showing normal levels of Col7 expression.

USAGE OF NON CULTURED EPIDERMAL GRAFTING FOR REVERTANT CELL THERAPY

We would like to acknowledge **José Duipmans** (Department of Dermatology) and **Dr. Mike Ruettermann** (Department of Plastic Surgery) from the University Medical Center Groningen for their involvement in this study.

After successful transplantation of revertant keratinocytes described in **Chapter 4**, we decided to investigate other methods to expand the revertant skin area without an *in vitro* cell culture. Non-cultured transplantation of epidermal cells has already been widely used in treatment of burn wounds, vitiligo and to some extent as therapy for chronic ulcers.[11-13] During one of the follow-up visits after the treatment described in **Chapter 5**, a new ulceration (8 cm^2) was found in the affected skin on the foot of patient 029-01 (Figure 3A). This patient had junctional EB, generalized intermediate subtype (JEB-gen-intermed), due to mutations in the *LAMB3* gene with multiple identified revertant skin patches.[14] We decided to treat this ulceration with transplantation of non-cultured epidermal cells in suspension. The ReCell® kit (Avita Medical) was chosen to provide certified equipment allowing preparation of a revertant cell suspension. According to the manufacturer, the ReCell® kit allows an expansion ratio up to 1:80. Because transplantation of non-cultured epidermal cells is an established treatment for chronic ulcers no approval from the ethical committee was neccesary.

The patient was admitted to hospital and wound debridement was performed. On the next day, a 1 cm^2 split thickness skin specimen was harvested with a dermatome from the same revertant skin patch as, used for treatment described in **Chapter 4**. The skin specimen was digested according to the ReCell® kit manual. Briefly, it was placed in the enzyme mixture provided by Avita Medical for 15 minutes and then scratched with the scalpel to allow epidermal and dermal cell isolation. Enzymes were then blocked by the blocking solution (Avita Medical) and the cell suspension was poured through a strainer to remove cell clumps. The obtained suspension was then placed in the syringe and applied onto the wound (Figure 3B) and onto the donor site. Dressings were placed on both locations and the first inspection took place seven days later during which no clinical improvement of the wound could be seen. The patient reported however, that the pain from the ulceration had substantially decreased.

The patient was seen further at two, four, six and ten weeks after the procedure and each time examination of the wound and photographic documentation were performed. During the follow-up visit at four weeks, signs of re-epithelialisation and reduction of erythema surrounding the ulcer were seen. At six weeks we observed a decrease in the wound area when a fully re-epithelialized bridge, splitting the original ulceration into two smaller wounds, was formed (Figure 3C). At ten weeks the two smaller ulcerations were still present and the patient reported

an increase of pain from the wound region. The decision not to take a follow up biopsy from the treated area to assess if transplantation of revertant cells succeeded was made. To accelerate healing we then chose to perform punch grafting, as was previously done in this patient (**Chapter 4**). Because of the impaired wound healing, most likely due to advanced age, usage of beta-blockers and arterial obstruction (toe brachial index: 0.71, ankle brachial index: 0.85), the first round of punch grafting was not successful. Within four weeks a slight reduction in size of both ulcers was achieved but no full re-epithelialization occurred. Over the course of the following 4 months, the patient chose a conservative treatment, consisting of dressings changes three times a week, which did not lead to improvement of the ulcers.

Seven months after the application of non-cultured revertant cells in suspension we saw that the fragment, that had re-epithelialized six weeks after the procedure, looked healthy. The two smaller ulcers had unfortunately grown larger in the direction of earlier untreated areas. The second round of revertant punch grafting, taken again from the patient's right shoulder was applied, which this time led to successful healing of the wounds.

In summary, partial re-epithelialisation after application of revertant cell suspension occurred. Unfortunately, analysis of the healed region for engraftment of lam-332 revertant keratinocytes was impossible, because we did not want to cause an iatrogenic ulceration with a new biopsy in an already affected region. The originally re-epithelialised area stayed blister free for at least 7 months, which might suggest that revertant cells were transplanted during the first procedure (Figure 3D). There was however, an unsuccessful attempt for transplantation of punch grafts in-between, which could have led to the engraftment of additional revertant keratinocytes in the area and positively affected the wound healing.

Transplantation of non-cultured epidermal cells in suspension could lead to successful enlargement of the revertant skin area, if the acceptor site is well prepared, in a patient without impaired wound healing. Further investigation of this technique is necessary in a small clinical trial setting, where the wound bed could be better controlled.

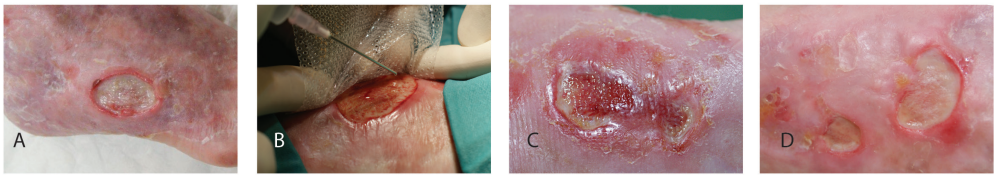

Figure 3. Transplantation of non-cultured epidermal cells in suspension harvested from the revertant skin on a chronic ulcer of JEB-gen-intermed patient. **A.** Chronic ulcer on patient's foot. **B.** Application of cell suspension isolated with ReCell® kit. **C.** Wound 6 weeks after the procedure. Partial re-epithelialization can be seen in the center of the wound. **D.** Wound 7 months after the procedure with expansion of the ulcerated area lateral. The part that was healed earlier (in C) remains stable.

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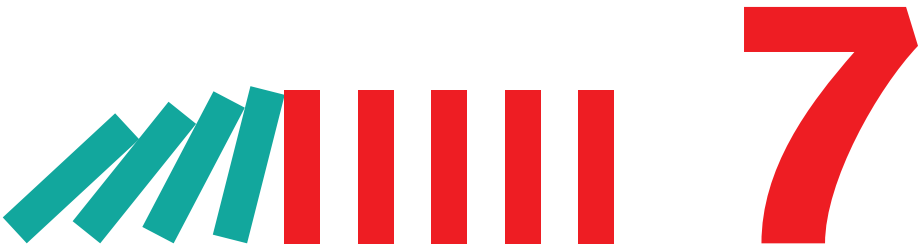

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

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Fragments of this chapter are part of the manuscript: Revertant cell therapy for inherited diseases, on the forefront of translational medicine, by Gostynski et al., in preparation.

REVERTANT CELL THERAPY: CURRENT STATE OF THE ART

How far are we?

The proof-of-principle of revertant cell therapy was demonstrated in **Chapters 2-4** of this thesis. Different techniques can be used to expand an area of healthy skin. In **Chapter 2** we showed that autologous transplantation of cultured epithelial sheets is feasible in a patient, although survival of revertant keratinocytes during graft production was insufficient. In **Chapter 3** we showed that production of cultured skin grafts could be improved and that long-term survival of revertant cells was possible in a mouse model. Finally, in **Chapter 4** we described the successful, long-term acceptance of revertant punch grafts was described as a cure of persistant ulcers in EB. Therefore, if a sufficient area of revertant skin is available, as in our patient with Lam-332 deficiency described in **Chapter 4**, it is currently possible to treat crucial affected areas with non-cultured autologous revertant skin grafts. This method is simple, does not require a special laboratory and is available to most patients with revertant mosaicism, depending on the size and localization of their healthy skin patch.

Advantages of the current approach

If one looks at the therapeutic approaches described in **Chapter 1** of this thesis, one will see that these are based on allogeneic material (protein therapy and cell therapy) or genetic manipulation (gene therapy). The most important value of revertant cell therapy is the use of autologous, naturally corrected cells.[1,2] It avoids the need to introduce allogeneic material, as in the cell therapy approaches, or to manipulate the genome, as required by gene therapy. Contrary to these methods, revertant cell therapy has no risk of inducing an autoimmune response or off-target effects in the genome that may induce neoplasia. As revertant cell therapy uses naturally corrected keratinocytes, it could be considered natural gene therapy combined with cell therapy.

Advantages	Disadvantages	Possible limitations
Autologous material	Limited number of revertant patients available	Depletion of revertant stem cells
No genetic manipulation	Limited expansion ratio of punch grafting	Higher mutation ratio in revertant cells
No auto-immune response		

Table 1. Advantages, disadvantages and possible limitations of the current approach to revertant cell therapy

Concerns and possible limitations of the current approach

As the advantages of revertant cell therapy seem to be indisputable; possible limitations and disadvantages should always be considered. First of all, revertant cell therapy can only be applied in patients with identified revertant mosaicism. At the moment it is expected that all of the JEB-gen intermed patients with mutations in *COL17A1* have visible revertant mosaicism and recently revertant mosaicism has been identified in a number of DEB patients.[3,4] We are, however, far from the statement that all patients with JEB and DEB have identifiable revertant mosaicism, as in many patients revertant patches are yet to be found. The age of patients can also limit the *in vitro* expansion, as it is normal for all humans to deplete the stem cell population with age.[5] Moreover, in the form proposed in **Chapters 2-4**, revertant cell therapy aims to only treat cutaneous manifestations of EB. With this being a disadvantage, as the treatment of the all affected organs should be the ultimate aim of a successful therapy for a genetic disease, one should not forget that most of the therapies currently under development are able to provide treatment of all the manifestations of EB.[6-10]

The biology of revertant mosaicism is not yet fully understood. For example it is believed that revertant keratinocytes are simply a healthy population of cells in an affected body and thus do not have a higher mutation rate than other corresponding cell populations.[11,12] Mutation rates in revertant cells have been determined in revertant mosaic patients with Wiskott-Aldrich syndrome (WAS), and was found to be normal,[12,13] which is a strong argument for the above stated assumption.

Depletion of the growth potential of revertant keratinocytes could also become a potential threat to revertant cell therapy. In the first successful gene therapy for EB, Mavilio et al. described problematic *in vitro* expansion of lam-332 deficient keratinocytes due to stem cell depletion.[14] These data suggest that because of extensive blistering, stem cells have to divide more often than in a healthy individual and thus their growth potential when found in cultured skin grafts is hereby impaired. One could speculate if the revertant cell population in EB patients becomes depleted and if this is a reason for in vitro growth disadvantage of revertant keratinocytes. In patients with WAS, X-linked SCID and adenosine deaminase deficiency exhibiting revertant mosaicism, a selective growth advantage *in vivo* of revertant lymphocytes has been observed.[15-18] If one assumes, that the same applies to revertant keratinocytes *in vivo*, the fact that revertant patches in EB patients seem to be stable in most of the patients after infancy could mean that the growth potential of revertant keratinocytes has already met their biological limit. When we take into account that *in vitro* keratinocytes can divide approximately 50 times,[19] which is in accordance with the Hayflick limit,[20] one could wonder if the difficulties in culturing revertant keratinocytes described in **Chapters 2 and 3** come from a specific growth advantage of Col17 negative keratinocytes *in vitro* and not from a depletion of growth potential of revertant cells.

The fact that long-term survival of revertant cells is achieved and that revertant cells have sufficient colony forming potential, **(Chapter 3)** together with the lack of blistering in revertant skin and success of transplantation of revertant skin **(Chapter 4)**, are strongly against depletion of revertant stem cells. Although both the higher mutation rate of revertant cells and depletion of revertant stem cells seem improbable, experiments to analyse the mutation rate and telomere length of revertant keratinocytes would certainly give clarification.

REVERTANT CELL THERAPY: EXPANDING CURRENT APPROACH

Future of revertant keratinocytes without in vitro expansion

In **Chapter 4** we presented the use of punch grafting for transplantation of revertant skin. An advantage of split thickness punch grafting lies in the small size of the biopsies that can easily be harvested from every part of the body leaving enough surrounding skin in the donor site to ensure healing with the remaining revertant skin. Patients with revertant mosaicism of the skin have single or multiple revertant patches, randomly distributed, with irregular shape and a diameter seldom larger than 5 cm, making the use of a dermatome to harvest traditional split skin grafts difficult. Furthermore, the aim to expand a revertant area means that the revertant donor site should also heal with the healthy epidermis. Harvesting a larger graft from one location might give the mutant cells an opportunity to repopulate the donor site.

The disadvantage of punch grafting is the relatively poor expansion ratio, which is typically not more than 1:4. The maximal treated area depends thus directly on the size of donor revertant patches. To achieve higher expansion from a single revertant patch, a different method of non-cultured epidermal grafting could be applied. Currently, when non-cultured epidermal grafting is considered, the highest expansion ratio can be achieved with the enzymatic digestion of small skin biopsies and application of isolated cells in suspension.[21-23] Such a method, proved successful in the treatment of burn wounds and vitiligo, can be applied during a single procedure in the operating theatre omitting the need for a GMP certified laboratory. In **Chapter 6** we described an attempt to treat a chronic ulcer in a JEB-gen-intermed patient with a commercial kit ReCell® (Avita Medical), which allows to digest a small skin biopsy and produce a skin cell suspension ready for transplantation.[24] The ReCell® manufacturer states that the kit allows an expansion rate of 1:80, meaning that from a 1cm^2 biopsy 80 cm^2 can be treated.[24] Our attempt described in **Chapter 6** was unsuccessful probably due to an impaired wound healing in the treated region. Approach with non-cultured epidermal cells in suspension should be however studied in the future in the more controlled environment. We believe that in combination with adhesive tape preparation of the wound bed, as described in **Chapter 2**, it will be an attractive solution for revertant cell therapy.

Towards successful in vitro expansion of revertant cells

In **Chapter 2** we showed the first attempt to expand revertant skin area with transplantation of cultured epidermal grafts in a patient affected by JEB-gen-intermed due to mutations in *COL17A1* and revertant mosaicism. Although the transplantation procedure and healing process were uncomplicated, we did not achieve restoration of the phenotype due to a low percentage of revertant keratinocytes in the graft. The original skin biopsy consisted of about 50% revertant cells and in the graft only 3% of the cells expressed Col17. In **Chapter 3** we again used a skin biopsy from the same patient, which also showed 50% of revertant keratinocytes at the start of the experiment. During the course of *in vitro* expansion and graft production, the percentage of Col17 revertant cells dropped to 20% and stayed at this level at least for 16 weeks after transplantation on the back of nude mice. It is therefore the *in vitro* phase that is responsible for the declining percentage of revertant keratinocytes. We will now speculate on the possible reasons for such shrinkage of the revertant population in experiments described in **Chapters 2 and 3**. With depletion of stem cells not being a convincing theory to explain the shrinkage of the revertant cell population relative to the mutant population *in vitro*, as explained earlier in this chapter, we looked into the influence of Col17 expression on keratinocytes. It has already been shown that Col17 deficient keratinocytes present a different phenotype *in vitro* in comparison to wild-type keratinocytes. First, the Col17 negative cells were found to have accelerated, but less directed, motility when compared to wild-type keratinocytes.[25,26] The recent study by Löffek et al. also shows that the migratory phenotype of Col17 negative cells is associated with enhanced phosphoinositide 3-kinase (PI3K) activity.[25] In 2006 Pankow et al. showed that PI3K is an important regulator of epidermal homeostasis and wound repair and that stimulation of Pl3K activity in cultured keratinocytes promoted cell proliferation.[27] Downstream from the Pl3K we find the NF-κB pathway. Van den Bergh et al. showed that Col17 deficiency causes a proinflammatory reaction in cultured keratinocytes driven by the NF-κB pathway and that Col17 deficient keratinocytes had higher levels of the NF-κB reporter than wild-type cells, suggesting higher stimulation of this pathway.[28] Not surprisingly, NF-κB is also found to be involved in epidermal homeostasis.[29] One could thus hypothesize that higher activity of Pl3K and NF-κB pathways in Col17 deficient cells than in the wild-type or revertant keratinocytes might be the reason for faster proliferation of Col17 mutant cells when cultured *in vitro*. Recently, during the 2014 congress of the European Society for Dermatological Research, Marsh et al. showed that siRNA Col17 knockdown keratinocytes show higher proliferation rates than wild-type cells giving an important argument supporting our theory.[30] Further investigation of revertant and mutant cells and their proliferation rate is needed, but in the future NF-κB inhibitors like Bay-11-7082,[28] might help to protect revertant keratinocytes during *in vitro* expansion from being overgrown by mutant keratinocytes. In **Chapter 6** we have presented our efforts to enrich the *in vitro* cell culture with Col17 revertant cells.

In 2007, in the study that aimed to asses the retroviral insertion safety for gene therapy of skin diseases, Larcher et al. was able to isolate a single holoclone and expand it *in vitro* to amounts needed for culture of skin equivalents.[33] Selection of a single Col17 revertant stem cell, a holoclone, could therefore lead to sufficient *in vitro* expansion to produce enough skin grafts to cover the whole human body. If possible, instead of one holoclone, more revertant cells with high proliferative potential could be selected by dilution of keratinocytes mass cultures combined with colony forming efficiency assay, as described by Larcher et al., to achieve not a monoclonal but a polyclonal revertant culture.[33] From personal communication with F. Larcher, we know that such selection is difficult and has not yet been achieved for revertant keratinocytes *(unpublished data)*.

Research on epidermal stem cells is evolving rapidly and, as described in **Chapter 1**, multiple keratinocyte stem cell markers have been already proposed. Visualisation and possible selection of revertant stem cells could help to establish a pure revertant cell culture, which would then allow production of fully revertant skin grafts. One of the options to show the number of revertant stem cells and their localisation would be to use biopsies from revertant skin and stain them with a stem cell marker. We have tested several monoclonal antibodies that are described in literature to stain epidermal stem cells. Worth mentioning are: DF1513 (CD71), GOH3 (integrin alpha 6),[34] A11b2 (integrin beta 1),[35] LHK15 and SPM190 (both keratin 15), 4A4 (p63)[36] and nb500 (survivin)[37] antibody. When stainings of normal human skin were analysed we discovered that there was no overlap between cell populations stained with those markers. It is possible that each of the antibodies visualized a stem cell rich population, however we were unable to pinpoint the revertant stem cells with immunofluorescence only. Further studies with new markers combined with colony forming efficiency assays and flow cytometry could help to establish an estimate of the revertant stem cell population. This will definitely add more to the understanding of revertant mosaicism as a phenomenon and in the more distant future could be translated to improve revertant cell therapy.

Other groups are also working on the development of revertant cell therapy. A clinical trial aimed at transplantation of cultured revertant skin grafts was registered by Stanford University on the www.clinicaltrials.gov between October 2011 and April 2014. It was, however, withdrawn prior to enrolment of the subjects and no data from this study are openly available. We hope that this thesis will encourage others to look for possible solutions to the problem of *in vitro* expansion of Col17 revertant cells. Ultimately, we believe that after future research, safe and successful selection techniques for Col17 revertant cells will be developed. In **Chapter 6** we showed an unsuccessful attempt to expand Col7 revertant cells because of the low amount of naturally corrected keratinocytes in the original biopsy. Such simple *in vitro* expansion of revertant keratinocytes isolated from patients with lam-332 or Col7 deficiency should be further studied, as there is currently no rationale to expect *in vitro* growth advantage of mutant cells.

REVERTANT CELL THERAPY: A NEW CONCEPT

Inducing pluripotent stem cells from revertant keratinocytes

Presuming that revertant skin grafts can be produced, *in vitro* expansion levels are often limited, for example by patient's age. Moreover, extracutaneous manifestations of EB, cannot be treated with transplantation of cultured epidermal grafts. To take full advantage of the presence of revertant keratinocytes, a combination of present knowledge with new developments in regenerative medicine is needed. The technique of inducing pluripotent stem cells (iPSCs) from a somatic cell, for which the Nobel Prize in Medicine in 2012 was awarded to John B. Gurdon and Shinya Yamanaka, can contribute to broaden the application of revertant mosaicism in therapy of all genetic diseases.[38-40] Cells with properties of an embryonic cell can be obtained from an already differentiated somatic cell by introduction of selected transcription factors (OCT4, c-MYC, KLF4 and SOX2).[39] IPSCs have been induced from skin fibroblasts, bone marrow derived mesenchymal cells and epidermal keratinocytes.[38,40-42] Induction of iPSCs from revertant tissue leads to patient specific, naturally corrected cells with theoretically unlimited expansion potential – a perfect tool for treatment of genetic diseases. Induction of pluripotent stem cells is at the moment widely performed with retroviral vectors. This is a very effective method, however safety concerns arise, as usage of viral vectors may be carcinogenic. Moreover, it is of importance to successfully differentiate IPSCs to target lineages to prevent teratoma formation. In order to find more clinically friendly approaches for iPSCs induction, the usage of transcription factors in the form of recombinant proteins, integration-free viral vectors or plasmid-based derivation could be applied.[43-45] Noteworthy is a very elegant strategy of synthetic mRNA introduction, which does not require integration within the genome and can be easily controlled owing to the quick degradation of mRNA.[46]

Recently iPSCs have been acquired from revertant skin of patients with EB. Tolar et al. isolated revertant keratinocytes from a healthy skin patch of a 10-year-old boy with severe generalized RDEB due to two loss-of-function mutations in *COL7A1*, paternal c. 3840delC and maternal g.6751-2A>G.[42] Keratinocytes isolated from the revertant patch showed the presence of the paternal mutation and, due to the skipping of exon 86, the maternal mutation was not present. This resulted in a shorter, but presumably functional *COL7A1* transcript. By using retroviral vectors, keratinocytes were reprogrammed into iPSCs, which then were differentiated into haematopoietic progenitor cells and keratinocytes. Our recent cooperation with Dr. Christiano's group from University of Columbia resulted in the induction of iPSCs from revertant keratinocytes isolated from a healthy skin patch of JEB-gen-intermed due to mutations in *COL17A1* gene.[47] This is the same patient that was described in 1997 by Jonkman et al. and is also involved in the research described in **Chapters 2 and 3** of this thesis. Revertant keratinocytes and therefore also iPSCs showed only the paternal mutation, while the maternal mutation was corrected due to

gene conversion. These cells were expanded *in vitro* and differentiated into keratinocytes, which could then form not only a 3D skin equivalent expressing wild type Col17, but also full thickness human skin in a murine model. Both studies were performed with introduction of retroviral vectors with required transcription factors. The next step would be to use more clinically applicable methods of iPSCs induction followed by *in vivo* testing in animal models, which would bring us closer to cultured revertant grafts obtained through induced pluripotent stem cells.

Systemic therapy with revertant cells – the ultimate goal

In **Chapter 1** we reviewed the usage of allogeneic stem cell transplantation for treatment of RDEB described in 2010.[48] Recently, a clinical study that uses transplantation of umbilical cord blood instead of bone marrow as a haematopoietic stem cell source combined with mesenchymal stromal cells following reduced intensity conditioning has started in the Netherlands and aims to achieve at least the same levels of disease amelioration, as achieved in the Minnesota trial, combined with improved safety and reduced mortality. Both Minnesota and Dutch studies are based on the fact that allogeneic stem cells that can express Col7, consist not only of haematopoietic stem cells but also of the Lin–/PDGFRα+ population that can transform to epidermal and dermal cells.[49] Tamai et al. not only characterized the Lin–/PDGFRα+ population, but also identified the SOS signal that mobilizes this population to regenerate injured epithelium. High mobility group box 1 protein (HMGB1) is highly expressed in patients with RDEB due to extensive blistering and therefore the presence of hypoxic keratinocytes. It is in response to these keratinocytes that HMGB1 can thus mobilize the Lin–/PDGFRα+ subpopulation of transplanted allogeneic stem cells to become Col7 expressing epidermal cells.[49] When Tolar et al. induced IPSCs from revertant keratinocytes; they also successfully differentiated them into the haematopoietic stem cells.[42] One could therefore imagine the autologous, naturally corrected haematopoietic stem cells containing Lin–/PDGFRα+ cells derived from revertant keratinocytes through induction of iPSCs and their subsequent auto-transplantation. Although such a "from skin to blood to skin approach" would still require certain conditioning prior to transplantation in order to create a niche, no graft versus host disease prophylaxis would be required. Revertant pluripotent cells could be expanded for this *in vitro* and differentiated into haematopoietic cells, mesenchymal and epidermal stem cells. This means that if needed, skin grafts could be produced to cover the whole body area of an EB patient or that systemic application of revertant haematopoietic stem cells would be possible.

With the induction of revertant iPSCs broadening the possible application of revertant cell therapy, we should not forget that this requires temporary genetic manipulation and therefore removes the earlier mentioned advantage of revertant cell therapy being free of artificial changes in the genome.

Translation of revertant cell therapy in EB to other genetic diseases

Revertant cell therapy in EB has received a lot of attention and interest. We believe that skin diseases will continue to be on the forefront of revertant cell therapy due to relatively easy measurement methods to monitor success of such therapy and the unique visual outcomes. Induction of iPSCs from revertant cells may also be used in other genetic diseases, such as WAS. One could easily speculate about iPSCs induced from revertant lymphocytes and transplantation of autologous haematopoietic stem cells in WAS. Currently therapy of WAS consists of protein replacement therapy, allogeneic stem cell transplantation or gene therapy, and therefore transplantation of autologous naturally corrected stem cells would remove the need for persistent change in genome of transplanted cells or graft versus host prophylaxis.[50] Not forgetting this theoretical advantage, it is too early to speculate if revertant cell therapy for WAS would be superior to therapies already being applied.

Revertant cell therapy: important element of personalized medicine for EB

In **Chapter 1** we discussed different approaches to treat EB. Recently, a newsletter issued by the EB patients organisation DEBRA reported that at Stanford University, USA, the first patient with RDEB was treated with transplantation of autologous skin grafts made from cells corrected by gene therapy.[51] Additionally, a clinical trial started in Salzburg, Austria for gene therapy in patients with JEB-gen-intermed has been reported in the same newsletter. The first RDEB patient has been enrolled in the aforementioned clinical trial of umbilical cord blood transplantation for EB in the Netherlands. As the many different therapies for EB mentioned in this thesis are becoming applicable, a personalized medicine approach to treat EB can be proposed (Figure 1). We believe that in the future, therapy for each EB patient will be considered separately after assessment of key factors: subtype of EB, type of mutation, age, extent of cutaneous and extracutaneous symptoms and presence of revertant mosaicism.

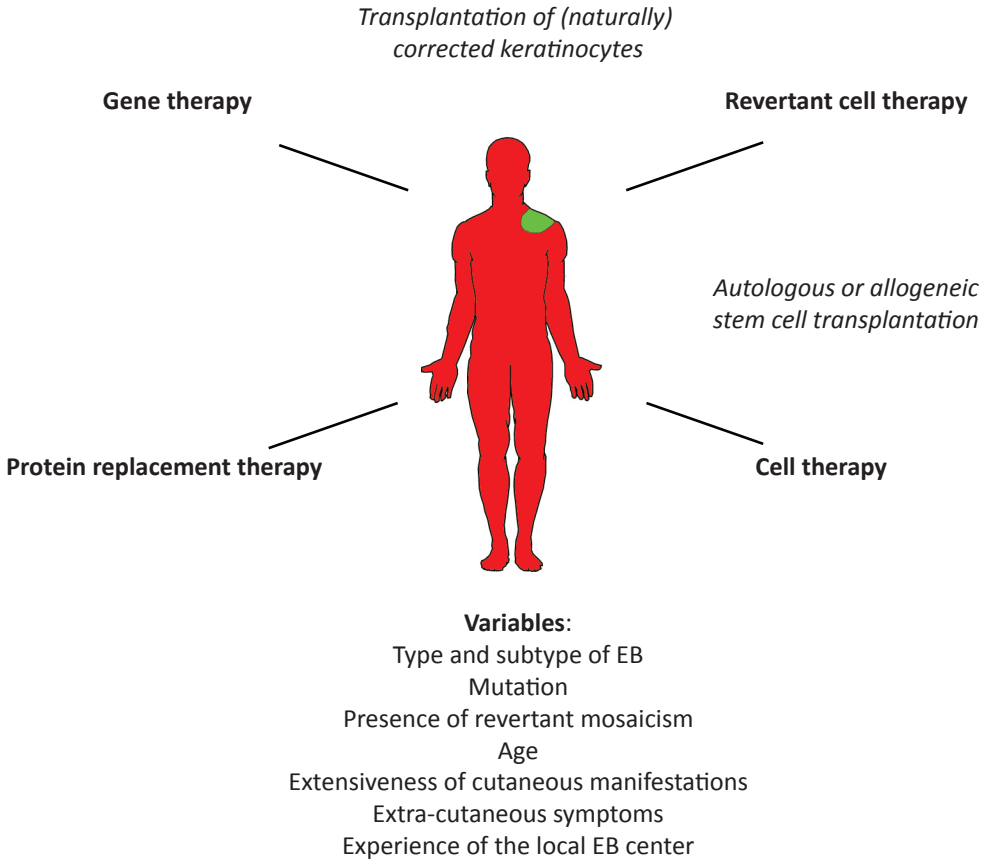

Figure 1. Schematic presentation of a future, personalized model of EB treatment with four major therapeutic approaches for EB in **bold** and with overlap between revertant cell therapy and cell therapy and gene therapy in *italic*.

REVERTANT *MOSAICISM*: A GOLD MINE FOR RESEARCH AND CLINICS

Discussion over perspectives for revertant cell therapy show the importance of the phenomenon of revertant mosaicism to the clinics. In addition to that, **Chapter 5** of this thesis shows how patients with revertant mosaicism can contribute to research on human biology.[52] In **Chapter 5**, we used reverse translation, from bedside to the bench, to analyse the hyperpigmentation of revertant patches in Col17 deficient patients. Although Tanimura et al. have already suggested the influence of Col17 on melanocytes in the murine model,[53] prior to the study described in this thesis no proof of such a fact had been shown in humans. Our study showed a significant correlation between the presence of melanocytes, pigmentation and Col17 expression. The model suggested by Tanimura, where paracrine stimulation of melanocyte stem cells by epidermal stem cells is missing when the latter expresses no Col17, may explain the dependence between Col17 deficiency and lack of melanocytes.[53] There are, however, other mechanisms that involve Col17, for example an increased proinflammatory response due to Col17 deficiency, as the earlier discussed study by Van den Bergh et al..[28] showed. Increased stimulation of interleukins 6 and 8 can lead to targeting of melanocytes by the immune system and inhibition of their growth.[54-56] As more studies are needed to further clarify these theories on reasons for melanocyte dependence on Col17 and possibly Lam-332, as described in **Chapter 5**, our observations and conclusions show that revertant mosaicism can be an important tool not only to develop successful therapies for EB, but also to perform research on this extremely interesting population of human knock-outs.

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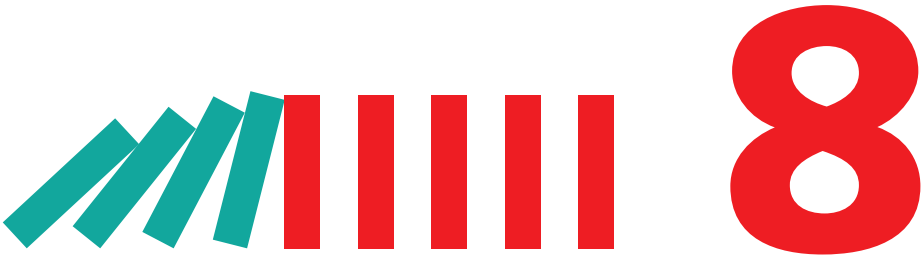

SUMMARY

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This thesis describes a quest to understand and use the phenomenon of revertant mosaicism for treatment of the heritable skin disease called epidermolysis bullosa (EB). EB is caused by mutations in the dermal and epidermal proteins and is characterised by blistering of the skin and mucosa.

In **Chapter 1** of this thesis we describe the pathological mechanism behind EB, we introduce the phenomenon of revertant mosaicism and discuss current approaches to treat EB. At the moment there are 18 genes identified in which mutations may cause EB. The recent consensus divides EB in four major types and more than 30 subtypes depending on the level of blister formation, affected protein, disease severity and presence of extra cutaneous manifestations. Moreover, a detailed overview of function of three proteins involved in the dermal epidermal junction, type XVII collagen (Col17), laminin-332 (lam-332) and type VII collagen (Col7) is given. Mutations in genes encoding Col17 and lam-332 cause junctional epidermolysis bullosa (JEB), while mutations in type VII collagen are responsible for the dystrophic variant of EB. At the moment no curative therapy is available for EB and the current approach to treat EB can be divided in three main groups: gene therapy, protein replacement and cell therapy. Gene therapy focuses on correction of the affected gene; protein replacement therapy aims at supplying the wild-type version of the affected protein, whereas cell therapy uses autologous or allogeneic cells to change the course of disease. The current state-of-the-art of those therapies is discussed in more detail in **Chapter 1**.

This thesis focuses on a novel approach to treat EB using revertant mosaicism. Revertant mosaicism (RM) is a phenomenon in which there is a co-existence of affected cells containing a disease-causing mutation (mutant), and cells in which the mutation is naturally corrected to the wild-type phenotype (revertant) within one individual . That is why RM is often addressed to as 'natural gene therapy'. Since the first description of RM in a patient affected with Lesch-Nyhan syndrome in 1988, this phenomenon has been found in many other diseases, like Wiskott-Aldrich Syndrom (WAS), Fanconi anemia and EB. There are different mechanisms described that can cause RM, like single base pair substitution or a second-side mutation. The full overview of those mechanisms can be found in **Chapter 1**. Within one individual many different correcting mechanisms can be found, for example 38 different reversions were identified in a patient affected with WAS.

In the skin RM was first described in 1995 in a patient affected by the JEB subtype caused by mutations in the *COL17A1* gene coding for the Col17 protein. Between affected skin areas on the patient's arms healthy looking skin patches were found. Immunofluorescence staining of the skin biopsy taken from a skin patch showed presence of Col17, whereas the biopsy taken from the affected skin was negative for the protein. The molecular mechanism behind RM in this patient was found in 1997. In cells with normal Col17 expression one of the original mutations causing the disease disappeared due to a gene conversion. The presence of healthy skin patches

was named revertant mosaicism and healthy cells were called revertant. Since 1997, RM has been found in many JEB and DEB patients and it is believed that all patients affected by mutations in *COL17A1* have revertant skin patches. Presence of the healthy cells within an affected body is a unique opportunity for autologous cell therapy. Different methods of skin transplantation have already been used to treat burn wounds and chronic wounds. Transplantation of revertant keratinocyte to expand the revertant skin area is the aim of revertant cell therapy.
In **Chapter 2** we describe an attempt to increase revertant skin area in a Col17 deficient, JEB generalized intermediate (JEB-gen-intermed) patient. The patient was a compound homozygous for a maternal frame shift mutation in exon 18 (c.1601delA) and paternal nonsense mutation in exon 51 (c.3676C>T). This patient had multiple revertant skin patches and in some of them the following correction mechanism was identified, i.e. part of the paternal allele of *COL17A1* containing a healthy copy of exon 18 was moved to the maternal allele and covered the c.1601delA mutation resulting in one allele with a paternal mutation and one wild-type. From such a naturally corrected revertant skin patch a skin biopsy was taken and cultured *in vitro* to acquire two 6x7 cm epidermal sheets. The wound bed was prepared by adhesive tape stripping – an innovative method that uses EB's pathological mechanism of reduced adhesion in the lamina lucida. Briefly, an adhesive tape was placed on the patient's thigh and small incisions were made around it. The lack of adhesion due to the mutations in *COL17A1* resulted in removal of the epidermis when tape was pulled off. Skin grafts were placed on the prepared wound bed and the healing process was uneventful and successful. Unfortunately, the functional test showed no reversion of EB phenotype and thus no expansion of revertant skin area. Analysis of the biopsy, cultured cells and epidermal grafts revealed that the percentage of revertant cells decreased from 50% in the biopsy to less than 3% in the graft. At that moment the reason for such a decrease was unknown.
Chapter 3 describes the animal model of the revertant cell therapy for EB. In this chapter we looked into survival of revertant cells during cell isolation, graft production and engraftment on immunodeficient mice. A biopsy from the same patient taken from the same revertant patch as in experiments described in **Chapter 2** was used. We isolated keratinocytes and fibroblasts and assessed the percentage of the revertant cells after the first passage to be 40%. This percentage dropped to 25% and 20% after the second passage and in the cultured skin equivalent, respectively. We grafted skin equivalents containing 20% of revertant cells on the immunodeficient mice and assessed the percentage of revertant area after 10 and 16 weeks. On both time-points 20% of the cells was revertant meaning that long-term survival of revertant keratinocytes *in vivo* is possible. In **Chapter 3** we also looked into the colony forming potential of revertant keratinocytes. We showed that revertant keratinocytes have a high ability to form colonies, but revertant colonies were smaller than mutant ones. This, together with the recently published work describing influence of Col17 on immunomodulation and NF-κB levels, gave us a basis to

formulate a theory about decrease of percentage of Col17 revertant cells during *in vitro* expansion. This theory is discussed in more detail in **Chapter 7**.

Chapter 4 describes a different approach to revertant cell therapy, where *in vitro* expansion of revertant cells is omitted and naturally corrected skin is transplanted directly onto the acceptor site. We successfully treated a then 69-year-old male patient affected by JEB-gen-intermed due to homozygous c.628G>A mutations in the *LAMB3* gene with revertant punch biopsy grafting. Multiple revertant patches on this patient's body were earlier identified. Immunofluorescence on a biopsy from his mutant skin showed strongly reduced staining for laminin-332, while normal levels of laminin-332 were observed in the revertant patches. In 2012 this patient presented with multiple, persistent (>1 year) ulcers. We used an earlier identified revertant skin patch on his right shoulder as a donor site and harvested 73 punch biopsy specimens that were then placed in the wounds. All biopsies were accepted and wounds healed within 2 weeks. During the course of 18 months no blistering or ulcerations were observed in the treated areas. Skin biopsies from both donor and acceptor site showed re-epithelialisation with revertant epidermis expressing normal levels of laminin-322. Thus, a successful expansion of revertant skin area and application of revertant cells in therapy of EB were shown.

Revertant mosaicism is a source of naturally corrected cells for therapy as well as an interesting phenomenon that allows studying two genetically different cell populations within one body. In **Chapter 5** we investigated why revertant skin patches in patients with revertant mosaicism and mutations in *COL17A1* are hyperpigmented in contrast to their mutant skin. In a population of 13 patients with EB and revertant mosaicism with mutations in *COL17A1* (n=8), *LAMB3* (n=2) and *COL7A1* (n= 3) the phenotype of affected (=mutant) and healthy (=revertant) skin was compared. The amount of pigment and density of melanocytes were identified in biopsies taken from mutant and revertant skin. There was a clinical difference in pigmentation in the *COL17A1* group, which was not present in the other two groups. Further, more pigment in the Fontana-Masson staining and a significantly higher melanocyte density was found in Col17 revertant skin versus Col17 mutant skin. In contrast, patients with mutations in *LAMB3* showed a lower melanocyte density and amount of pigment in both revertant and mutant skin. Mutations in *COL7A1* did not to have a correlation with the amount of pigment or density of melanocytes. In the study presented in **Chapter 5** we concluded that pigmentation depends on Col17 and that lam-332 might have a negative influence on proliferation of melanocytes and therefore on pigmentation. How the proteins of the dermal-epidermal junction regulate the pigmentation and density of melanocytes is still unknown. Col17 seems however to have an important role in the inflammatory pathways, cell signalling and survival of melanocyte stem cells, while lam-332 might influence the melanocytes proliferation. These theories will have to be investigated in the future.

Many other hypotheses and possible improvements to expand the revertant area of the skin were investigated during work on this thesis. In **Chapter 6** three selected experiments, that were perhaps not a direct step forward on the path to revertant cell therapy but are worth mentioning, are presented. First, an approach to select Col17 revertant keratinocytes involving differences in adhesion between Col17 positive and negative cells is described. Unfortunately, not only Col17 is responsible for adhesion to plastic and coatings such as lam-332 or type I collagen in keratinocytes and therefore we could not separate keratinocytes based on Col17 expression. Next, the successful sorting of living revertant and mutant Col17 cells with flow cytometry is shown using the 233 monoclonal antibody. Non-enzymatic detachment of keratinocytes was used in this method to preserve the extracellular domain of Col17 and allow immunofluorescence staining. This method allowed further *in vitro* culture of sorted cells. This is, however, not possible to apply in the clinical setting at the moment due to safety regulations.

In the second part of **Chapter 6** we describe an attempt to establish a murine model for Col7 revertant cell therapy based on a similar approach as presented in **Chapter 3**. A recessive dystrophic EB patient due to mutations in the *COL7A1* gene had a revertant patch on her arm. From this patch a biopsy was taken, cells were isolated and skin equivalents were cultured. In the side samples only less then 10% of cells were revertant while in the biopsy taken previously from this patch 85% of the epidermis showed presence of Col7. Skin grafts were placed on the immunodeficient mice and analysed with immunofluorescence after 10 and 16 weeks. No revertant cells could be found in the grafts meaning that we did not succeed in transplantation of healthy cells. Low numbers of naturally corrected cells in the original biopsy taken for cell isolation was a probable cause of the unsuccessful experiment. Col7 revertant cell therapy should be further studied with a biopsy containing a higher percentage of revertant keratinocytes.

The last experiment presented in **Chapter 6** involves the same patient as described in **Chapter 4**. He underwent transplantation of non-cultured epidermal cells in suspension harvested from his revertant skin patch to treat a chronic leg ulcer. Impaired wound healing due to age and arterial obstruction had a negative influence on the procedure and complete re-epithelialization was not reached. We expected that some revertant cells were engrafted with this procedure, because a bridge dividing the ulcer in two smaller wounds was seen 6 weeks after the procedure and remained stable during the seven-month follow-up. A study of transplantation of non-cultured revertant cells in suspension with a controlled, well prepared acceptor site, will validate this technique for future usage.

Chapter 7 of this thesis summarizes the findings of **Chapters 2-6** and discusses advantages and disadvantages of revertant cell therapy as an approach to cure EB. Currently, treatment of limited skin areas with punch grafting is possible. In the near future we expect to use new techniques to transplant revertant cells with and without *in vitro* expansion, depending on the needs of the

patient. **Chapter 7** also discusses the future use of induced pluripotent stem cell technology to produce a population of naturally corrected stem cells that could be used to treat EB locally (with skin grafts) and systemically (through infusion).

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9

SAMENVATTING

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Dit proefschrift weerspiegelt de zoektocht naar het begrijpen en gebruiken van revertant mozaïcisme bij de behandeling van de erfelijke huidziekte Epidermolysis Bullosa (EB). EB wordt veroorzaakt door verschillende mutaties in genen van epidermale hechtingseiwitten en wordt klinisch gekenmerkt door blaarvorming van de huid en slijmvliezen.
In **Hoofdstuk 1** van dit proefschrift beschrijven we de pathologische mechanismen achter EB. Daarnaast wordt het begrip revertant mozaïcisme geïntroduceerd en bespreken we de huidige stand van zaken van onderzoek naar behandelingen van EB. Op dit moment zijn er 18 genen geïdentificeerd welke, wanneer gemuteerd, tot EB kunnen lijden. Op basis van de huidige consensus wordt EB onderverdeeld in vier hoofdtypen en meer dan 30 subtypen. De classificatie vindt plaats op basis van het niveauvan blaarvorming in de huid, de ernst van de ziekte, het aangetaste hechtingseiwit en de mutatie in het betrokken gen. Tevens geven we een gedetailleerd overzicht van de functies van de drie eiwitten die betrokken zijn bij de epidermale verbindingen in de huid. Deze eiwitten betreffen type XVII collageen (Col17), laminine-332 (lam-332) en type VII collageen (Col7). Mutaties in genen die coderen voor Col17 en lam-332 veroorzaken junctionele EB (JEB), terwijl mutaties in Col7 verantwoordelijk zijn voor de dystrofische variant van EB (DEB). Tot op heden bestaat er geen genezing voor EB. Momenteel worden er meerdere therapieën onderzocht en deze kunnen onderverdeeld worden in drie hoofdgroepen: gentherapie, eiwitvervangingstherapie en celtherapie. Gentherapie is gericht op correctie van het aangetaste gen, eiwitvervangingstherapie probeert de 'wild-type' vorm van het desbetreffende eiwit in het lichaam te introduceren, terwijl celtherapie autologe of allogene cellen gebruikt om de functie van de gemuteerde cellen over te nemen.

Dit proefschrift richt zich op een nieuwe methode om EB te behandelen, waarbij gebruik gemaakt wordt van het fenomeen van revertant mozaïcisme. Revertant mozaïcisme (RM) is een co-existentie van zieke (mutante) cellen en gezonde (revertante) cellen in één organisme. RM wordt ook vaak aangeduid als 'natuurlijke gentherapie'. Na de eerste beschrijving van RM bij een patiënt met de ziekte van Lesch-Nyhan in 1988 werd dit fenomeen ook gesignaleerd bij het Wiskott-Aldrich Syndroom (WAS), Fanconi anemie en EB. Er zijn verschillende mechanismen beschreven die verantwoordelijk zouden kunnen zijn voor RM, zoals een vervanging van enkele basenparen of een tweede mutatie. Het volledige overzicht van deze mechanismen is te vinden in **Hoofdstuk 1**. Bij aangedane patiënten kunnen veel verschillende correctiemechanismen voorkomen. Zo werden 38 verschillende reversies gevonden bij een patiënt met WAS.
De presentatie van RM in de huid werd voor het eerst beschreven in 1995 bij een patiënt met JEB. Tussen aangedane huidgebieden op de arm van deze patiënt werd gezond uitziende huid gevonden. Immunofluorescentiekleuringen van huidbiopten van gezond uitziende huid toonden aanwezigheid van Col17, terwijl de huidbiopten van de aangedane huid negatief waren voor

dit eiwit. De moleculaire verklaring volgde in 1997. De patiënt bleekmutaties in het *COL17A1* gen te hebben dat codeert voor het type XVII collageen (Col17) eiwit. In de cellen met normale Col17 aankleuring bleek één van de twee recessieve mutaties verdwenen door een genconversie. Deze huidcellen werden revertant genoemd, en de aanwezigheid van normale plekken huid werd revertant mozaizicme gedoopt. Sinds 1997 is RM gevonden bij veel JEB en DEB patiënten, en wordt aangenomen dat bij alle patiënten die zijn aangedaan door mutaties in *COL17A1* sprake is van revertante plekken. De aanwezigheid van gezonde cellen in een aangedane patiënt is een unieke kans voor autologe celtherapie. In het verleden zijn verschillende werkwijzen gebruikt voor huidtransplantaties in de behandeling van brandwonden en chronische wonden. Het doel van revertante celtherapie is om door middel van transplantatie van revertante keratinocyten het revertante huidoppervlak te vergroten.

In **Hoofdstuk 2** beschrijven we een poging om het revertante huidoppervlak te vergroten bij een patiënt met gegeneraliseerde intermediare JEB (JEB-gen-intermed) door Col17 deficiëntie. De patiënt had een maternale 'frame shift' mutatie in exon 18 (c.1601delA) en een paternale 'nonsense' mutatie in exon 51 (c.3676C>T). Deze patiënt had meerdere gebieden van revertante huid. In een aantal van die gebieden werd onderstaand correctiemechanisme geïdentificeerd. Een deel van het paternale allel van *COL17A1*, welke een gezonde kopie van exon 18 bevatte, was verplaatst naar het tweede chromosoom. Daardoor werd de c.1601delA mutatie gecorrigeerd, wat resulteerde in een allel met een paternale mutatie en een 'wild-type' allel. Een huidbiopt werd genomen van een natuurlijk gecorrigeerde revertant gebied op de arm. Dit werd vervolgens *in vitro* gekweekt om twee 6x7 cm epidermale transplantaten te verkijgen. Het wondbed werd voorbereid met "adhesive tape stripping" - een innovatieve methode die het pathologische mechanisme van EB gebruikt van verminderde adhesie in de lamina lucida. Kort gezegd werd er tape geplaatst op de dij van de patiënt en werden vervolgens rondom kleine incisies gemaakt. Het gebrek aan adhesies door de mutaties in *COL17A1* resulteerde in loslating van de epidermis bij het lostrekken van de tape. De huidtransplantaten werden op het gepreppareerde wondbed geplaatst en het genezingsproces verliep succesvol en ongecompliceerd. De functionele test toonde helaas geen reversie van het EB fenotype en dus ook geen uitbreiding van het revertante huidoppervlak. Analyse van de huidbiopten, gekweekte cellen en epidermale huidtransplantaten liet een daling zien van het percentage revertante cellen tot 50% in het huidbiopt en tot minder dan 3% in het huidtransplantaat. De reden voor een dergelijke daling was op dat moment onbekend.

Hoofdstuk 3 beschrijft een diermodel voor de revertante celtherapie voor EB. In dit hoofdstuk hebben we gekeken naar de overleving van revertante cellen tijdens celisolatie, transplantaatproductie en transplantatie op immuundeficiënte muizen. Er werd een biopt gebruikt uit hetzelfde revertante gebied van de in **Hoofdstuk 2** beschreven patiënt. Van de geïsoleerde keratinocyten en fibroblasten bleek het percentage revertante cellen na de eerste passage 40% te zijn. Dit percentage daalde tot 25% en 20% na respectievelijk de tweede passage en in de (uiteindelijke) gekweekte huidequivalenten. We transplanteerden de huidequivalenten met 20% revertante cellen op de immuundeficiënte muizen en bepaalden na 10 en 16 weken het percentage revertante huidgebieden. Op beide meetpunten was er nog steeds 20% revertante cellen aanwezig, dit betekent dat langdurige overleving mogelijk is van revertante keratinocyten *in vivo*. Tevens werd het kolonievormende potentieel van revertante keratinocyten beschreven. Revertante keratinocyten bleken een hoge capaciteit te hebben om kolonies te vormen, echter waren deze kolonies kleiner van grootte dan de door de mutante cellen gevormde kolonies. In combinatie met recent gepubliceerd werk dat de invloed van Col17 op immunomodulatie en NF-kB niveaus beschrijft, gaf deze bevinding ons een uitgangspunt om een theorie over het terugbrengen van het percentage Col17 revertante cellen tijdens *in vitro* expansie te formuleren. Deze theorie wordt inhoudelijk beschreven in **Hoofdstuk 7**.

Hoofdstuk 4 beschrijft een andere benadering van de revertante celtherapie, waarbij *in vitro* expansie van revertante cellen wordt overgeslagen en natuurlijk gecorrigeerde huid direct getransplanteerd kan worden naar de acceptorplaats. Met succes hebben wij een 69-jarige patiënt behandeld door middel van revertante biopttransplantatie. Deze patiënt had een JEB-gen-intermed als gevolg van een homozygote (c.628G>A) mutaties in het *LAMB3* gen. Al eerder werden bij deze patiënt meerdere revertante huidgebieden geïdentificeerd. Immunofluorescentiekleuring op een huidbiopt van de mutante huid toonde een sterk verminderde aankleuring voor lam-332, terwijl in de revertante huid een normale aankleuring voor lam-332 werd waargenomen. In 2012 presenteerde deze patiënt zich met meerdere, chronische ulcera die meer dan 1 jaar bestonden. We gebruikten een eerder geïdentificeerd revertant huidgebied op zijn rechter schouder als donorplek en oogsten 73 huidbiopten die werden teruggeplaatst in de ulcera. Alle biopten groeiden in, en de ulcera genazen binnen 2 weken. In de daaropvolgende 18 maanden werden geen nieuwe blaren of ulceraties in de behandelde gebieden waargenomen. Huidbiopten van de donor- en de acceptorplaats toonden re-epithelisatie, waarbij de revertante epidermis normale aankleuring voor lam-332 liet zien. Dit onderzoek toonde dat bij de behandeling van EB door middel van toepassing van revertante cellen een succesvolle uitbreiding van het revertante huidoppervlak verkregen kan worden.

Revertant mozaïcisme is een bron van natuurlijk gecorrigeerde cellen voor behandeling en is een interessant fenomeen omdat dit het mogelijk maakt om twee genetisch verschillende celpopulaties binnen het lichaam te bestuderen. In **Hoofdstuk 5** onderzoeken we waarom revertante huid gehyperpigmenteerd is bij patiënten met revertant mozaïcisme en *COL17A1* mutaties, in tegenstelling tot de mutante huid. We vergeleken het fenotype van mutante en revertante huid in een populatie van 13 patiënten met EB en revertant mozaïcisme met mutaties in *COL17A1* (n = 8), *LAMB3* (n = 2) en *COL7A1* (n = 3). In de afgenomen huidbiopten van zowel revertante als mutante huid hebben we de hoeveelheid pigment en de dichtheid van melanocyten bestudeerd. In de *COL17A1* groep werd een klinisch verschil in pigmentatie gevonden, welke niet aanwezig was in de andere twee groepen. In Col17 revertante huid vonden we meer pigment in de Fontana-Masson kleuring, daarnaast was de dichtheid van melanocyten significant hoger in de groep met revertante huid ten opzicht van de groep met mutante huid. Patiënten met mutaties in *LAMB3* toonden echter een lagere dichtheid van melanocyten en de hoeveelheid pigment in zowel de revertante als de mutante huidgebieden. Mutaties in *COL7A1* hadden geen correlatie met de hoeveelheid pigment of dichtheid van melanocyten. Wij concludeerden uit de studie in **Hoofdstuk 5** dat de pigmentatie afhankelijk is van Col17 en dat lam-332 een negatieve invloed zou kunnen hebben op de proliferatie van melanocyten en daarmee op pigmentatie. Het is nog onbekend hoe de eiwitten in de epidermale basaalmembraan zone de pigmentatie en dichtheid van melanocyten reguleren. Col17 lijkt echter een belangrijke rol te spelen bij ontstekingsreacties, communicatie tussen cellen en de overleving van de stamcellen van melanocyten, terwijl lam-332 de proliferatie van melanocyten zou kunnen beïnvloeden. Deze hypothese zou in de toekomst verder onderzocht moeten worden.

Tijdens mijn promotie-traject werden ook andere theorieën en hypothesen getest om uitbreiding van het revertante huidoppervlak te bereiken. In **Hoofdstuk 6** worden drie experimenten beschreven die niet een succes zijn geweest, maar mogelijk een belangrijke rol spelen bij de verdere ontwikkeling van revertante celtherapie. In het eerste gedeelte wordt een poging gedaan de revertante en mutante cellen te scheiden op basis van een mogelijk verschil in de aanhechtingskracht. Helaas hebben keratinocyten meer aanhechtingseiwitten dan alleen Col17 en bleken de gebruikte krachten niet voldoende om een verschil te laten zien tussen beide celpopulaties. Daarnaast werd er een protocol ontwikkeld om levende keratinocyten met het 233 monoclonaal antilichaam gericht tegen Col17 aan te kleuren en vervolgens te scheiden door het gebruik van flow cytometrie. Helaas is flow cytometrie vanwege veiligheidsmaatregelen niet geschikt voor klinische toepassing, maar deze kan mogelijk verdere experimenten met revertante keratinocyten vereenvoudigen.

Het tweede deel van **Hoofdstuk 6** bespreekt een poging om de experimenten van **Hoofdstuk 3** te herhalen met Col7 revertante cellen. Een biopt werd genomen van een revertant huidgebied van de onderarm van een patiënte met recessieve dystrofische EB (RDEB) door mutaties in het *COL7A1* gen. Cellen werden geïsoleerd en gekweekt, waarna huidtransplantaten werden gemaakt en getransplanteerd op muizen zoals beschreven in **Hoofdstuk 3**. In het biopt dat eerder van dezelfde patiënte afgenomen is, was 85% van de epidermis revertant. In de kweek van het nieuwe biopt was slechts 10% van de cellen revertant. In de op de muizen getransplanteerde huid werden na 10 en 16 weken geen revertante cellen gevonden. Wij denken dat het lage percentage van revertante cellen in het biopt een oorzaak was voor het niet succesvol verlopen van het experiment. Daarom zou Col7 revertante celtherapie verder onderzocht moeten worden.
Het laatste experiment dat beschreven wordt in **Hoofdstuk 6** betreft een transplantatie van niet-gekweekte keratinocyten-in-oplossing bij een patiënt eerder beschreven in **Hoofdstuk 4**. Een chronisch ulcus cruris van deze patiënt werd behandeld met een oplossing van epidermale cellen, geoogst uit zijn revertante huidgebied op de rechterschouder met behulp van een ReCell Kit®. Helaas werd complete genezing niet bereikt. De meest waarschijnlijke oorzaak is slechte wondgenezing door de hogere leeftijd en het onderliggend arterieel vaatlijden. Wij denken dat er een deel van de getransplanteerde cellen wel degelijk is ingegroeid, omdat het oorspronkelijke ulcus zes weken na de ingreep in tweeën verdeeld werd door gezond ogende huid. Dit gezond ogende huidgebied bleef stabiel tijdens de 7 maanden durende follow-up. Om antwoord te geven op de vraag of deze methode verder gebruikt kan worden voor revertante celtherapie is een klinische trial nodig met een goed voorbereide acceptor plek.
Hoofdstuk 7 van dit proefschrift geeft een overzicht van de bevindingen uit de **Hoofdstukken 2 t/m 6** en bespreekt de voordelen en nadelen van revertante celtherapie als een behandeling om EB te genezen. Momenteel zijn we in staat om een beperkt huidoppervlak te behandelen door middel van revertante biopttransplantaties. In de nabije toekomst hopen we nieuwe technieken in te kunnen zetten om revertante cellen te transplanteren met en zonder *in vitro* expansie, afhankelijk van de behoeften van de patiënt. Daarnaast wordt het toekomstige gebruik van geïnduceerde pluripotente stamceltechnologie besproken, zodat een populatie van natuurlijk gecorrigeerde stamcellen gebruikt zou kunnen worden om EB plaatselijk (met huidtransplantaten) of systemisch (via infuus) te behandelen.

9

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APPENDICES

ACKNOWLEDGEMENTS

My journey from Warsaw to Groningen started when, as a 4th year medical student, I moved to the Netherlands to work on an exciting project, which turned into this PhD. These years have been challenging, interesting, rewarding and wonderful. I achieved many personal goals, including completing this book, and many people helped me get to where I am now. I would like to take this opportunity to mention them here.

First I would like to thank my promotor Prof. dr. Marcel Jonkman and co-promotor Dr. Marjon Pasmooij.

Marcel, thank you for your courage to appoint a young medical student from Poland. Without your inspiration, time and expertise, this book would never have been written. Thanks to you, the researcher inside me had a chance to develop. Thank you for your enthusiasm, stimulating discussions and your guidance. You introduced me to the world of dermatology and EB and gave me a lot of freedom. I very much look forward to taking on the next challenge together.

Marjon, even before you became my official co-promotor you were always there, asking if things are going well and if you could help. During all these years, your input and comments were always helpful and stimulating. Your thoroughness in checking my manuscripts and presentations will have my admiration for years. Aside from work, I very much enjoyed all our discussions over drinks at the many conferences and meetings!

Dear members of the reading committee, Prof. dr. D.R. Roop, Prof. dr. J. Schalkwijk and Prof. dr. P.M.N. Werker, thank you for the time invested in reading and reviewing my thesis. Your critical comments made this book better.

International collaboration was very important for chapters 3, 5 and 6 of this thesis and for the development of new techniques that are currently being used to push revertant cell therapy forward. This could not have been done without the following people:

Prof. Del Rio and Dr. Larcher, Marcela and Fernando, thank you for your invitations to Madrid and co-operation that resulted in 2 publications in this book. My gratitude also goes to Marta Garcia, Mariajo Escamez and the rest of CIEMAT lab for the work done together. I hope that this is not the end of our collaboration.

Dr. Meana, Alvaro, thank you for your hospitality in Oviedo. Thanks to you, I not only learned a lot about the production of skin grafts but also how beautiful the north of Spain is. Sara Llames and Eva Garcia, thank you for your involvement in the projects. It is always a pleasure to visit Oviedo and your laboratory.

Prof. Dennis Roop I would like to thank you for making my stay in Denver possible and Anya Bilousova together with Igor Kogut for making it a very fruitful few weeks. The techniques, which I learned during my time in Colorado, are already bringing results.

I would like to express my gratitude for our EB patients, their families and the EB patient organizations, especially Vlinderkind and DEBRA Nederland for their involvement and support of my research.

The whole international EB community had an important influence on my work through all the stimulating congresses.

Dr. Pas - my introduction to the laboratory techniques would not be possible without you, Hendri. Your daily supervision meant a lot, especially in the first years of my work in Groningen. Your ability to ask the right question often made me look at the problem from the different angle.

Miranda, thank you for your time invested in teaching me cell culture and not forcing me to read all the SOPs. Especially at the beginning, you definitely helped me understand the Dutch culture better.

I am very grateful to the whole Dermatology lab for your input in this work and excellent technical assistance. Janny and Gonnie, you helped me a lot with immunofluorescence and preparation of slides. Guus, Duco and Laura, your help with many different techniques was also crucial.

José Duipmans, for your commitment to the cause and for being such a great person to work with.

Gilles Diercks, for always sharing your expertise in dermatopathology. Thank you for your input in Chapter 5!

The PhD students I worked with: my roommates, Gerda and Mira and then Angelique and Laura, I thank you for a great time together and for accepting the chaos on my desk; the EB team, Marieke, Wing-Yan, Peter, Jeroen, Kasia, Iana for sharing a passion; and the rest of the skin researchers, Ena, Joost, Ineke, Janine, Laura, Wianda, Tanja, - I learned a lot from you all and enjoyed time spent together.

The dermatologists from the dermatology department in the UMCG; I would like to thank you all for your supervision during residency, as I am able to learn a lot from each one of you. I would especially like to thank Dr. Horvath and Dr. Schuttelaar for allowing me to combine research with clinics in the last few months and hopefully in the future.

All the dermatology residents, present and past, I would like to thank for your help, support and friendship.

Co-workers of the Department of Dermatology I am grateful for support, help with my experiments, work and studies.

I very much appreciate that dermatologists in the MCL, Leeuwarden understood that during the past half year of my residency there, my mind was sometimes focused on this thesis and not on our daily practice.

My paranimfs, Jeroen and Joost, thank you for standing next to me during my defense and helping with the preparations. Let the march of the penguins begin!

Maarten and Joost, thank you for the hours spent on my Dutch summary.

Martijn Wolf, for the layout of this book and your last minute help.

Friends. You all know who you are. It is a pleasure to have such a great friends, as you are. You helped me feel at home in Groningen, stayed in touch from all parts of the world and were there for me, when I needed you the most. Thank you!

Family. You gave me a lot of support and time but what is most important, is that you were always interested in my work. Thank you for that. My parents in-law I have to especially thank for sending my lovely wife to study in Warsaw, so we could meet. My grandmother I am very much grateful to you for cheering me on throughout this project.

My mom, thank you for always being there, your support and colorful discussions; without you I would not be here. You are the best mom a son could ask for.
Kasia, thank you for sharing your life with me, for all the moments together, in the past and in the future. You are the love of my life.
Wiktoria, you have changed my life for the better in so many ways.
I am the luckiest man with both of you in my life.

Antoni

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Sci Transl Med 2014, accepted for publication.

CURRICULUM VITAE

Antoni Henryk Gostynski was born on June 5th, 1984 in Lublin, Poland. He was raised in Warsaw, Poland, where he completed Czacki high school, graduating in a mathematic-physics profiled class. Directly after high school he was accepted to study medicine at the I Medical Faculty of the Medical University of Warsaw.

Four years into his studies, he undertook a challenging PhD project on revertant cell therapy for epidermolysis bullosa under the supervision of Prof. dr. Marcel F. Jonkman and Dr. Marjon Pasmooij at the University of Groningen, the Netherlands. Within the first two years of the project, next to research, he learned the Dutch language and was accepted to complete his medical degree at the University of Groningen. After obtaining his medical degree in January 2012, he continued to work on experiments contributing to this thesis. The work done by Antoni, finalized and ongoing, consisted of many fruitful collaborations with researchers in the Netherlands, Spain, Poland and the USA.

In April 2013 Antoni began his residency in dermatology and venereology at the University Medical Center Groningen. He continues to divide his time between clinical training, research and his family. At the end of 2014 this thesis was finalized. Antoni aims to complete his residency in 2018. During this time he hopes to stay involved in research on EB.

Currently Antoni lives in Groningen with his wife Kasia and daughter Wiktoria.